Aspects of the Control of Polyphosphoinositide Metabolism by ADP-Ribosylation Factors and Diacylglycerols

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Abstract

In recent years, polyphosphoinositides, particularly phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$) and phosphatidylinositol-3,4,5-trisphosphate ($PI(3,4,5)P_3$), have become increasingly important due to the discovery that these lipids, rather than just having calcium or growth factor signalling functions, play key roles in a wide range of other important cellular processes. For this reason, their generation and control is tightly regulated by a growing group of enzymes that produce them, degrade them or depend on their presence to function.

One of the main enzymes involved in $PI(4,5)P_2$ production is Type I phosphatidylinositol 4-phosphate 5-kinase (PI(4)P5K), which generates it by phosphorylation of PI(4)P. $PI(4,5)P_2$ is in the central fork of phosphoinositide cascades, from where several second messengers are generated. Its production by Type I PI(4)P5K is regulated, amongst others, by members of the small GTP binding protein family of ADP-ribosylation factors or ARFs. Part of my thesis has concentrated on determining the isoform selectivity between ARFs and lipid kinases, and the finding that ARF6, the most divergent isoform of the six known ARFs, is the preferred regulator. Previous studies have always defended the indiscriminate activation by both ARF1 and ARF6. This is the first time that a clear preference for ARF6 is proved *in vitro* and supported in permeabilised cell-systems.

The activity of Type I PI(4)P5K has been known to be influenced by the presence of certain lipids, which either activate it, phosphatidylserine and phosphatidic acid, or inhibit it, as it happens by its own product, $PI(4,5)P_2$. I have found that another important cell signalling lipid, diacylglycerol, is also capable of activating the lipid kinase.

Finally, one of the functions of $PI(4,5)P_2$ is the generation of the second messenger $PI(3,4,5)P_3$, by the subsequent phosphorylation of the inositol ring by phosphoinositide 3-kinases (PI3Ks). This lipid is involved in a very large number of crucial events in the cell, such as cell migration, cell survival and phagocytosis, amongst others. For the first time, I have found that ARF proteins are also involved in the generation of PIP_3, by activating one of the four known isoforms of PI3K, namely p110γ.

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Abbreviations

APS: Ammonium Persulfate
ARF: ADP-Ribosylation Factor
ATP: Adenosine-5-triphosphate
BFA: Brefeldin A
BSA: Bovine Serum Albumine
BTK: Bruton's tyrosine kinase
$CaCl_2$: calcium chloride
°C: degree Celsius
C2 domain: PKC homology domain 2
DAG: 1.2-diacylglycerol
DGK: Diacylglycerol Kinase
DMPC: Dimyristoyl Phosphatidylcholine
DTT: DL-Dithiothreitol
EDTA: Ethylenediamine tetraacetic acid
EGFR: Epidermanl Growth Factor Receptor
EGTA: ethylene glycol-bis (β-aminoethyl ether) N, N, N' N'-tetra acetic acid
ER: Endoplasmic Reticulum
fMLP: N-formylated-Methionine-Leucine-Phenylalanine tripeptide
FPLC: Fast Protein Liquid Chromatography
GAP: GTPase activating protein
GEF: Guanine Exchange Factor
GFP: Green Fluorescent Protein
GPCRs: G-protein Coupled Receptors
GST: Glutathione-S-Transferase
GTP: Guanosine-5-Triphosphate
GTPγS: Guanosine-5'-O-(3-thiotrisphosphate)
HCl: hydrochloric acid
Hepes: N-[2-hydroxyethyl]piperzine-N'-[2-ethanesulfonic acid]
HRP: horseradish peroxidase
IP_3: Inositol-1,4,5-trisphosphate
IPTG: isopropyl-d-d-thiogaltopyranoside
LB: Luria Broth
MgATP: Magnesium adenosine triphosphate
min: minutes
$MgCl_2$: magnesium chloride

MVE: Multivesicular Endosome
MW: molecular weight
NaCl: sodium chloride
NaH_2PO_4: sodium phosphate monohydrate
$(NH_4)_2SO_4$: ammonium sulphate
NaN_3: sodium azide
$NaHCO_3$: Sodium bicarbonate
Ni-NTA: nickel-nitrilotriacetic acid
NMT1: *N*-myristoyltransferase-1
PA: phosphatidic acid
PAGE: Polyacrylamide Gel Electrophoresis
PBS: Phosphate-buffered Saline
PC: Phosphatidylcholine
PCR: Polymerase Chain Reaction
PDGF: Platelet-derived Growth Factor
PDK1/2: 3-phosphoinositide-dependent protein Kinase 1/2
PE: Phosphatidylethanolamine
PEG: Polyethylene Glycol
PH domain: Pleckstrin Homology domain
PI: Phosphatidylinositol
PIs: Phosphoinositides
PIPK: Phosphatidylinositol Phosphate Kinase
PI(3)P/PI(4)P/PI(5)P: Phosphatidylinositol-3/4/5-phosphate
PIP_2/PI(4,5)P_2: Phosphatidylinositol-4,5-bisphosphate
PIP_3/PI(3,4,5)P_3: Phosphatidylinositol-3,4,5-trisphosphate
PITP: Phosphatidylinositol Transfer Protein
PI3K: Phosphoinositide 3-kinase
PI4K: Phosphoinositide 4-kinase
PI(4)P5K: Phosphatidylinositol 4-phosphate 5-kinase
PIPES: Piperazine-N, N'-bis[2-ethanesulfonic acid]
PKA: Protein Kinase A
PKB/Akt: Protein Kinase B
PKC: Protein Kinase C
PKD/PKCμ: Protein Kinase D/Cμ
PLC: Phospholipase C
PLD: Phospholipase D
PMA: Phorbol 12-myristate 13-acetate
PMSF: phenylmethanesulfonyl fluoride
PS: Phosphatidylserine
psi: pounds per square inch
PTEN: Phosphatase and tensin homology
PX domain: Phox homology domain
RBD: Ras-binding Domain
ROCK: Rho Kinase
RTKs: Receptor Tyrosine Kinases

SDS: Sodium Dodecyl Sulphate
SH2/3 domains: Src Homology 2/3 domains
SEM: Standard Error of the Mean
SHIP1/2: Src homology 2-containing inositol phosphatase 1/2
SLO: Streptolysin-O
TEMED: N, N, N' , N'-Tetramethylethylenediamine
TBS: Tris-buffered Saline
TGN: *trans*-Golgi Network
TLC: Thin Layer Chromatography

Chapter One

Introduction

1.1 PHOSPHOINOSITIDES

Eukaryotic cells and their internal organelles such as the endoplasmic reticulum, the nucleus or the mitochondria, are surrounded by dynamic structures called membranes. These structures are active barriers which delimit and compartmentalise the cell and its interior, they can move, its components are degraded and resynthesised and they have their own identity and specific composition depending on the cell type and organelle. The main components of membranes are lipids, primarily phospholipids, cholesterol and sphingolipids. But other organic structures add complexity to the lipid bilayer, especially proteins and carbohydrates.

But as well as having such crucial structural function, membranes and more precisely some of the lipids that compose the bilayer, have other more complex and not less vital functions such as being part of the signalling network of the cell. From the moment that an external signal arrives to the outer surface of the cell until the ultimate stages where the cell responds for example by secreting, dividing or locomoting towards or away from a signal, these lipids are involved in the transduction of the signalling cascades. Their presence, generation and degradation are tightly controlled and myriads of different proteins and enzymes depend on them to interact with and transfer onwards the message to produce the ultimate biological effect. Some of these lipids are the source of second messengers or they are second messengers themselves, i.e. diffusible low-weight molecules which relay a signal within the cell. Defects in their metabolism can lead to disorders and serious diseases such as cancer.

One of these very important groups of lipids are phosphoinositides (PIs). PIs are found in unicellular eukaryotic organisms and thus must have appeared quite early in evolutionary terms. They are found in low abundance if compared

with the rest of phospholipids, between 5 and 10% of total membrane lipids. Nevertheless, recognition of their importance has been growing over the last 20 years since the discovery that they were the origin of the key second messengers diacylglycerol (DAG), inositol-1,4,5-trisphosphate (IP_3) and phosphatidylinositol-3,4,5-trisphosphate (PIP_3). On the whole, these phospholipids are connected to two basic cell physiological events, namely membrane fusion/vesicular transport and signal transduction by hormones or neurotransmitters into the cell.

Structure

The structure of the phosphatidylinositol (PI) molecule is a simple one and no more complicated than that of other phospholipids. It is composed of the typical phospholipid diacylglycerol backbone, the two acyl chains bound to carbons 1' and 2' of the diacylglycerol, and the phosphate group bound to carbon 3' and to the inositol ring forming a phosphodiester bond, as seen in Figure 1.1. However, the position and number of phosphate groups added to the 5 free hydroxyl groups of the inositol ring allows for up to 37 different theoretical conformations. In living cells however, only positions 3', 4' and 5' of the inositol ring have been found to be reversibly phosphorylated by the combined action of various kinases and phosphatases and therefore, 8 different phosphoinositides can be found in cellular membranes. The most common phosphoinositide is PI, which constitutes at least 80% of total

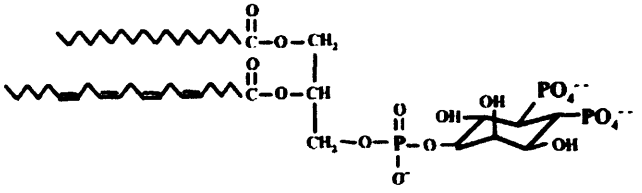


Figure 1.1 Structure of the molecule 1-stearoyl, 2-arachydonoyl, *sn*-Glycero-3-[Phosphoinositol-4,5-bisphosphate]

phosphoinositides. PI(4)P and PI(4,5)P_2 are next, but they are still only 0.5% of total lipids in cellular membranes. The other 5 are PI(3)P, PI(5)P, PI(3,4)P_2, PI(3,5)P_2 and PI(3,4,5)P_2 (Rameh and Cantley, 1999; Anderson *et al.*, 1999). The last three are especially scarce in cellular membranes, but their levels can rise suddenly upon stimulation (Vanhaesebroeck *et al.*, 2001). Figure 1.2 shows the different enzymes involved in the sequential phosphorylation and hydrolysis of the different phosphate groups that takes place in mammalian cells (Payrastre *et al.*, 2001; Takenawa and Itoh, 2001; Abel *et al.*, 2001; Vanhaesebroeck *et al.*, 2001).

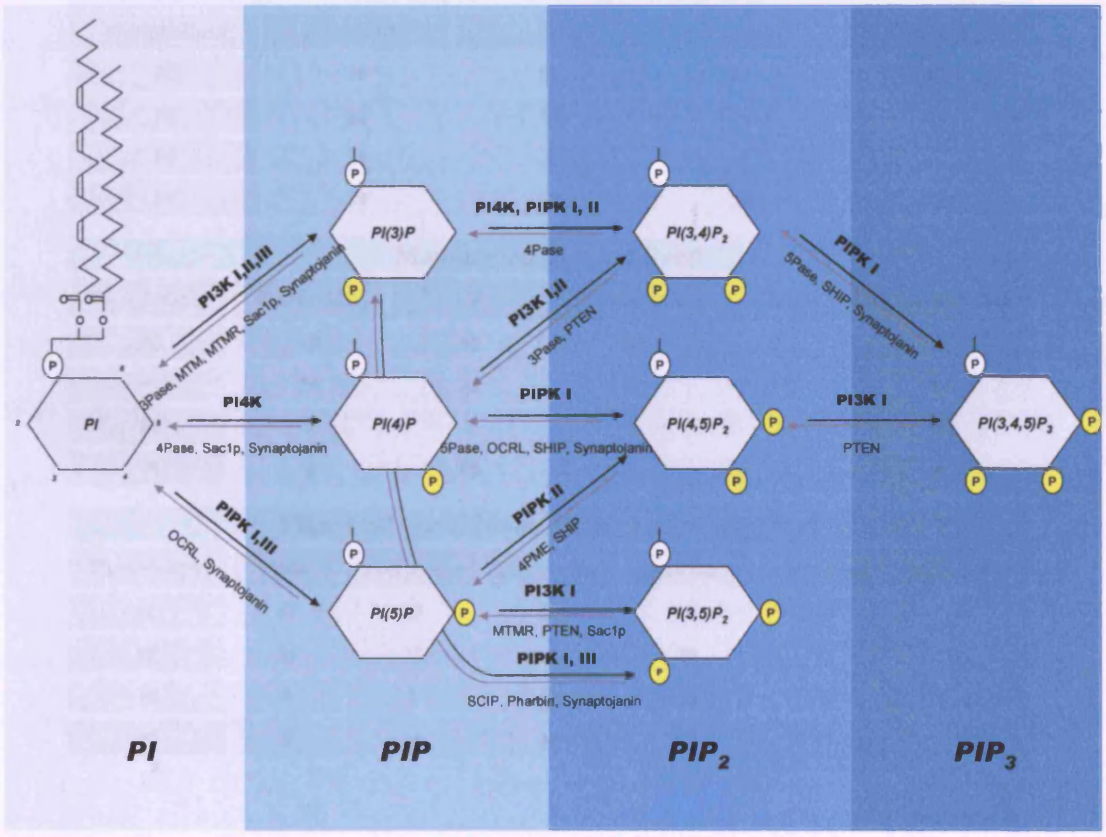


Figure 1.2 Pathways of phosphoinositides synthesis and interconversion.
The figure shows the pathways for the biosynthesis of the eight existent phosphoinositides and the majority of known kinases and phosphatases involved in their generation. Some of the pathways have only be proven to take place *in vitro* but not *in vivo*.

General Localisation

Phosphoinositides are found in practically all eukaryotic endomembranes (see Table 1.1). Nonetheless, the distribution of PIs in general on the plasma membrane is asymmetrical, for it is only on its inner leaflet that these phospholipids can be found as individual freely diffusing molecules, like phosphatidylserine and the majority of phosphatidylethanolamine (Verkleij and Post, 2000).

Yeast Cell (*Saccharomyces cerevisiae*)				
Phospholipid	Plasma Mb.	ER	Vacuole	Mitochondrium
PC	18	50	51	40
PE	22	24	21	27
PS	36	9	4	3
PI	**19**	**12**	**20**	**15**

Mammalian Cell (rat liver)					
Phospholipid	Plasma Mb.	ER	Lysosome	Golgi	Mitochondrium
PC	40	60	48	51	44
PE	24	23	17	21	34
PS	9	2	3	6	1
PI	**8**	**10**	**6**	**12**	**5**

Plant Cell (cauliflower, castor and mung bean)						
Phospholipid	P.Mb.	Microsome	Chloroplast	Peroxisome	Tonoplast	Mitochond.
PC	47	47	55	54	47	44
PE	35	31	6	29	32	34
PS	8	2	n.d.	2	5	n.d.
PI	**5**	**14**	**14**	**10**	**11**	**7**

Table 1.1 Lipid composition of subcellular fractions of yeast, mammalian and plant cells. The table shows the percentage of the four major constituents of living membranes of eukaryotic cells against total amount of phospholipids (Daum and Vance, 1997).

Moreover, the PI-distribution on the plasma membrane seems to be enriched in certain domains known as **lipid rafts** or **caveolae**. These microdomains have reduced lateral mobility, a chemical composition distinct from the rest of the membrane, high in sphingolipids and cholesterol, and variable thickness (Binder *et al.*, 2003). They are surface areas abundant in molecules involved in intracellular signal transduction, for instance, G-protein coupled receptors, Src family tyrosine kinases, and endothelial nitric oxide synthase (eNOS) (Smart *et al.*, 1999). An agonist-sensitive pool of PIs, namely PI(4)P, PI(4,5)P_2 and PI(3,4,5)P_3 as well as PA, has been described in these lipid microdomains (Pike and Casey, 1996; Liu Y. *et al.*, 1998; Waugh *et al.*, 1998; Bodin *et al.*, 2001), although in the case of PI(4,5)P_2 at least, this is a controversial statement (Watt *et al.*, 2002). The inner leaflet of rafts is enriched in saturated phosphoinositides with structural function. However, PIs have almost exclusively polyunsaturated acyl chains, 30-80% composed of a stearoyl and arachidonoyl fatty acyl pairing in mammalian cells (Mahadevappa and Holub, 1982; Holbrook *et al.*, 1992). Hence PIs, which are quantitatively minor lipids in these microdomains, are not backbone components of rafts but rather signalling molecules capable of rapidly interacting and recruiting specific effectors to generate a signal.

Functional pools of PIs can also be found in **intracellular membranes** together with their metabolising enzymes. Examples of this can be seen in yeast vacuoles, the enzyme VPS35 (yeast PI3K) and the phosphoinositide PI(3)P (Odorizzi *et al.*, 2000); the trans-golgi network and PI3K-C2α in mammalian cells (Domin *et al.*, 2000), as well as PI4K III and PI(4)P5K, which are recruited to the Golgi through an ARF1-dependent mechanism to generate PI(4)P and PI(4,5)P_2

(Godi *et al.*, 1999). PI(3)P and PI(3,5)P_2 are selectively concentrated on early and late endosomes respectively (Odorizzi *et al.*, 2000; Birkeland and Stenmark, 2004).

Phosphoinositides are also present in the membrane of the **nucleus**, and they go through similar synthesis and hydrolysis processes as they do in the other membranes. However, there is another pool of phosphoinositides within the nucleus that does not form part of the lipid bilayer and whose metabolism is regulated independently from that present elsewhere in the cell. Although these PIs generate second messengers such as DAG and IP_3, it is becoming increasingly clear that in the nucleus PIs may act by themselves to influence pre-mRNA splicing and chromatin structure (Martelli *et al.*, 2004). For instance, PI(4,5)P_2 has been detected in subnuclear domains containing pre-mRNA processing factors identified as nuclear speckles, and the absence of membranes in these structures suggests that nuclear PIs are actually presented to their metabolising enzymes in a non-membranous form, probably bound to proteins (Boronenkov *et al.*, 1998; Anderson *et al.*, 1998).

Discrete Localization and Function

PIs are not distributed at random in the cell. Each stereoisomer has a unique distribution within cellular membranes and its localisation is spatially and temporally controlled, as well as having particular and specific functions (Odorizzi *et al.*, 2000). Those PIs directly involved in membrane traffic are represented in Figure 1.3.

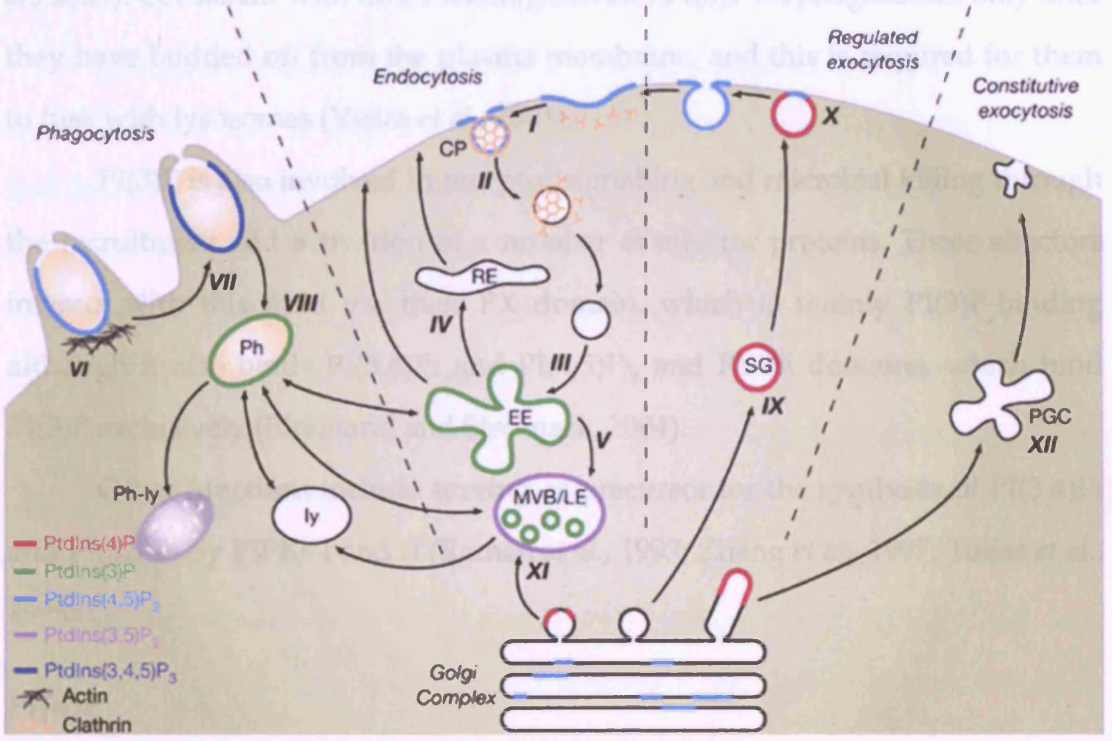


Figure 1.3 **Phosphoinositide composition of subcellular membranes of the endocytic and exocytic pathways.**
The numbers represent the different steps involved in each one of the processes. CP – *coated pits*; EE – *early endosome*; RE – *recycling endosome*; MVB/LE – *multivesicular body/late endosome*; Ly – *lysosome*; Ph – phagosomes; Ph-ly – phagolysosome; SG – *secretory granule*; PGC – *post-Golgi carrier* (De Matteis and Godi, 2004).

PI(3)P

Phosphatidylinositol-3-phosphate is principally found in the cytoplasmic leaflet of endosomes and of phagosomes, being the most abundant phosphoinositide in these internal membranes, around 4% of total lipids. This lipid is probably generated on the surface of early endosomes and not on the incoming vesicles and this difference may be used to give directionality to the fusion reaction and to recruit the endosomal sorting machinery (Roth, 2004). Thus, a possible role of PI(3)P would be to identify the destination membrane for fusion of incoming vesicles to give directionality to the fusion reaction (Rubino *et*

al., 2000). Consistent with this, PI3KIII generates PI(3)P on phagosomes only after they have budded off from the plasma membrane, and this is required for them to fuse with lysosomes (Vieira *et al.*, 2001).

PI(3)P is also involved in receptor signalling and microbial killing through the recruitment and activation of a number of effector proteins. These effectors interact with this lipid via their PX domain, which is mainly PI(3)P-binding although it also binds PI(3,4)P_2 and PI(4,5)P_2, and FYVE domains, which bind PI(3)P exclusively (Birkeland and Stenmark, 2004).

Other functions include serving as precursor for the synthesis of PI(3,4)P_2 and PI(3,5)P_2 by PIPKs I and II (Rameh *et al.*, 1997; Zhang *et al.*, 1997; Tolias *et al.*, 1998a).

PI(4)P

Phosphatidylinositol-4-phosphate is one of the most abundant PIs, constituting 93% of total cellular monophosphate derivatives of phosphatidylinositol (PIP) in NIH3T3 cells, against 5% of PI(3)P and 2% of PI(5)P (Rameh *et al.*, 1997). It is particularly abundant in the Golgi, where it accounts for the majority of present PIs, around 5% of total lipids (Roth, 2004). PI(4)P is the main precursor of PI(4,5)P_2 synthesis. In yeast, membrane traffic through the Golgi requires PI(4)P generated by Pik4 and does not require PI(4,5)P_2 (Audhya *et al.*, 2000). In mammalian cells, PI(4)P functions in maintaining Golgi structure both directly and through conversion to PI(4,5)P_2, probably by recruiting cytoskeleton to Golgi membranes, by targeting the AP-1 adaptor protein complex, and by maintaining the flux of membrane moving through the organelle (Wang *et al.*, 2003).

PI(5)P

Phosphatidylinositol-5-phosphate has been one of the last PIs to be discovered and due to its low levels and difficulties on determining its presence and relative abundance, its functions are still not absolutely clear. However, there is now enough preliminary evidence to confirm that it is a signalling molecule in its own right. Its levels have been reported to increase in platelets in response to thrombin stimulation (Morris *et al.*, 2000), and to decrease in mouse 3T3-L1 fibroblasts and adipocytes upon hypo-osmotic shock (Sbrissa *et al.*, 2002). PI(5)P has also been reported to exist and function in the nucleus (Clarke *et al.*, 2001). The PHD finger of the tumour suppressor ING2, a motif common in many chromatin-regulatory proteins, specifically interacts with PI(5)P and was proposed to function as a nuclear PI(5)P receptor to regulate nuclear responses to DNA damage (Gozani *et al.*, 2003). Finally PI(5)P has also been identified as a novel key intermediate for insulin signalling in F-actin remodelling and GLUT4 translocation (Sbrissa *et al.*, 2004).

PI(3,4)P_2

Phosphatidylinositol-3,4-bisphosphate is mostly found in the plasma membrane, but it is also present in multivesicular bodies and in the nuclear envelope (Watt *et al.*, 2004). This bisphosphate derivative of phosphatidylinositol (PIP_2) is generated in cells by two opposing pathways: D5 dephosphorylation of PI(3,4,5)P_3 by SHIP1 and SHIP2 (Leslie *et al.*, 2001) and D3 phosphorylation of PI(4)P by Class II PI3K (Rameh and Cantley, 1999) or D4 phosphorylation of PI(3)P. In an integrin-signalling pathway in platelets, PI(3,4)P_2 was found to be generated from PI(3)P by an unidentified phosphoinositide 4-kinase independently of PI(3,4,5)P_3 (Banfic *et al.*, 1998), and also upon induction of

peroxide-induced oxidative stress (Gray *et al.*, 1999; van der Kaay *et al.*, 1999). $PI(3,4)P_2$ seems to be involved in the activation pathway of PKB/Akt. Both PKB and PDK1 (a kinase which phosphorylates PKB on Thr308 to fully activate it) bind to $PI(3,4)P_2$ and $PI(3,4,5)P_3$ with similar affinities *in vitro*, albeit it is not clear which one of the two is involved in their activation *in vivo*. A third undetermined kinase, termed PDK2, has been reported to also bind to $PI(3,4)P_2$ and hence phosphorylates PKB on Ser473 and activate it in conjunction with PDK1 (Scheid *et al.*, 2002). $PI(3,4)P_2$ levels have also been found to rise due to oxidative stress, with the consequent activation of PKB (van der Kaay *et al.*, 1999).

$PI(3,5)P_2$

Phosphatidylinositol-3,5-bisphosphate was first discovered in yeast subjected to osmotic shock and then detected in mammalian cells (Dove *et al.*, 1997; Whiteford *et al.*, 1997). Fab1 in yeast and PIKfyve in mammals are the PI(3)P5Ks in charge of the production of $PI(3,5)P_2$. The invagination of multivesicular endosomes (MVE) and late endosomes requires the production of $PI(3,5)P_2$ (Odorizzi *et al.*, 1998, 2000). PI(3)P, and presumably $PI(3,5)P_2$, incorporated into the internal vesicles of MVEs is hydrolysed when these vesicles are transferred to the lumen of lysosomes or the yeast vacuole (Wurmser and Emr, 1998). This PI is also involved of the recruitment of myotubularin to the late endosomal compartment, and it is involved in epidermal growth factor receptor trafficking from late endosome to lysosome (Tsujita *et al.*, 2004).

$PI(4,5)P_2$

Phosphatidylinositol-4,5-bisphosphate has been shown to be maintained at relatively constant levels in cells (McLaughlin *et al.*, 2002), but some agonists for cell surface receptors such as growth factors and components of the

extracellular matrix cause rapid but modest changes in cellular $PI(4,5)P_2$ content (Auger *et al.*, 1989; Weernink *et al.*, 2000). This lipid second messenger is mainly found in the plasma membrane (Balla T. and Varnai, 2002; Meyer and Teruel, 2003) but it is also present in several other cellular organelles such as the endoplasmic reticulum, the nuclear envelope or the Golgi apparatus (Watt *et al.*, 2002; Balla and Varnai, 2002). Since the discovery that $PI(4,5)P_2$ was the precursor of the second messengers DAG and IP_3 some twenty years ago, a plethora of different functions have been discovered which imply that this particular polyphosphoinositide is involved in membrane traffic, the arrangement of the actin cytoskeleton and several other processes which are summarised hereafter.

Precursor of Second Messengers. Hydrolysis of $PI(4,5)P_2$ by the phospholipase C family, on the perception of suitable stimulus, generates the two second messengers DAG and IP_3. DAG is involved in the activation of protein kinase C (PKC), via binding to its C1 domain. PKC actively participates in a diversity of signalling pathways that control cell proliferation, differentiation, survival, transformation and apoptosis (see PKC section). Nonetheless, DAG also participates in the activation of several other proteins containing the same binding domain, such as RasGRP1, DGKα, β and γ, Munc13-1, PKD and others (Brose and Rosenmund, 2003). IP_3 is responsible for a transient increase in intracellular free Ca^{2+} (Nishizuka *et al.*, 1986; Berridge and Irvine, 1989).
Another very important second messenger is $PI(3,4,5)P_3$ which is generated in response to extracellular signals by Class I PI3Ks by D3-phosphorylation of $PI(4,5)P_2$ (Hawkins and Jackson, 1992; Rameh and Cantley, 1999).

Regulation of the Actin Cytoskeleton. Actin remodelling is directly or indirectly involved in many vital cell functions, such as maintenance of modulation of cell shape, motility, cytokinesis, the formation of filopodia, lamellipodia, membrane

ruffles and phagosomes, endo and exocytosis, mRNA localization and growth regulation (Doughman *et al.*, 2003a). The actin cytoskeleton has also been implicated as both a target and a mediator of signal transduction initiated through receptor tyrosine kinases and extracellular matrix-integrin systems (Hsuan *et al.*, 1998). The activities of many factors involved in regulating the dynamics of actin filaments are controlled by $PI(4,5)P_2$. $PI(4,5)P_2$ causes the dissociation of G-actin from actin-monomer-binding proteins, as well as the uncapping of the actin filament barbed ends; in addition, it enhances both actin filament crosslinking and the linkage of actin filaments to the plasma membrane and modulates the activity of some of these proteins by inducing conformational changes or regulating their interaction with other cytoskeletal or membrane proteins (Sechi and Wehland, 2000). The most important ones are:

a. **Profilin.** This protein functions by sequestering actin monomers as a complex termed profilactin. $PI(4,5)P_2$ disrupts this interaction, allowing for actin polymerisation at sites of high $PI(4,5)P_2$ concentration. It also binds $PI(3,4)P_2$ and $PI(3,4,5)P_3$ (Lassing and Lindberg, 1988; Lu *et al.*, 1996).

b. **Gelsolin.** $PI(4,5)P_2$ has also been proposed to promote actin polymerisation by interfering with the binding of capping proteins to filament ends. The actin severing/capping protein gelsolin is displaced from the barbed ends of actin filaments by the phosphoinositide, permitting rapid actin polymerisation (Hartwig *et al.*, 1995; Barkalow *et al.*, 1996). Gelsolin belongs to a Ca^{2+}-regulated family of F-actin/binding proteins, which includes villin, severin, CapG and scinderin. These proteins are also known to be regulated by PIPs and PIP_2 by an antagonistic mechanism to Ca^{2+} (Hsuan *et al.*, 1998).

c. **Vinculin.** Involved in focal adhesions, $PI(4,5)P_2$ disrupts the existing intramolecular interaction between the head and tail domains of vinculin,

resulting in the exposure of its actin, α-actinin, talin, paxillin, VASP, ponsin, vinexin and PKC binding sites (Bailly, 2003). Past studies have revealed that vinculin plays a central role in mechanical coupling of integrins to the cytoskeleton, as well as in the control of cytoskeletal mechanics, cell shape, protrusion amplitude and cell motility. Vinculin could be the long-sought missing link that bridges the adhesion scaffold and the actin polymerisation machinery in the focal complexes, thus specifically targeting actin polymerisation to the leading edge of the cell to sustain lamellipodia extension (Critchley, 2000).

A clear example for the involvement of $PI(4,5)P_2$ in focal adhesions is the specific localisation of PI(4)P5Kγ to these structures, where it binds to the FERM domain of talin, as β-integrin does by mediation of $PI(4,5)P_2$. Talin is a component of focal adhesions that couples β-integrin cytodomains to F-actin, as well as providing a scaffold for signalling proteins (di Paolo *et al.*, 2002; Ling *et al.*, 2002; Barsukov *et al.*, 2003).

d. WASP/N-WASP and ERM Proteins. WASP, and its more widely expressed homologue N-WASP, have emerged as central node proteins that regulate actin polymerization in response to multiple upstream signals. $PI(4,5)P_2$ provokes conformational changes in the protein that in synergy with Cdc42, induce actin assembly. This output activity of N-WASP is mediated through the actin-related protein 2/3 (Arp2/3) complex, a seven-protein complex capable of stimulating actin filament nucleation (Sechi and Wehland, 2000; Prehoda and Lim, 2002). ERM proteins act in a very similar way to WASP proteins, where interaction with $PI(4,5)P_2$ also activates the protein by inducing conformational changes and localises it to the plasma membrane. They also work in synergy with a small G protein, Rho, which influences the

phosphorylation state, activation and localisation of ERM proteins at the plasma membrane. ERM proteins interact with membrane components such as CD44, CD43 and intercellular adhesion proteins (ICAMs). Because of their ability to bind F-actin in vitro, ERM proteins have been considered *bona fide* candidates for mediators of interactions between the actin cytoskeleton and the plasma membrane (Sechi and Wehland, 2000).

Exocytosis. $PI(4,5)P_2$ has been found to be necessary for Ca^{2+}-dependent regulated exocytosis both in neuro-endocrine cells and in haematopoietic cells.

a. **Neuro-endocrine cells.** There are two types of vesicles in these mammalian cells which store neurotransmitters, chemical mediators and peptide hormones: dense core secretory granules and synaptic vesicles. PITPα and PI(4)P5Kγ have been purified as cytosolic factors that can prime the cells for subsequent stimulation with Ca^{2+} and successive exocytosis of the first type of granules, in conjunction with a secretory granule-resident PI4K (Hay *et al.*, 1995; Wiedemann *et al.*, 1996). Two proteins that contain two Ca^{2+}-binding C2 domains, synaptotagmin and rabphilin 3A, an effector of the small GTPase Rab3A, can bind $PI(4,5)P_2$ in a Ca^{2+}-dependent manner, and it is envisaged that this interaction, along with interaction with SNARE molecules involved in the docking/fusion process, are responsible for the exocytosis of synaptic vesicles (Cockcroft and de Matteis, 2001).

b. **Haematopoietic cells.** Cells of the immune system are capable of secreting proteins, cytokines and histamine readily upon detection of infectious agents. Degranulation in mast cells and neutrophils is dependent not only on a rise of intracellular levels of Ca^{2+} but also on the presence of a small G protein, identified as ARF (Fensome *et al.*, 1996; Way *et al.*, 2000) or Rab3D (Roa *et al.*, 1997; Tuvim *et al.*, 1999). ARF, and also PITP, seem to trigger secretion by

increasing the levels of $PI(4,5)P_2$, both directly by activating PI(4)P5K and indirectly by activating PLD, which in turn produces PA, a reported *in vitro* activator of Type I PI(4)P5Ks (Cockcroft and de Matteis, 2001).

Endocytosis. Clathrin-mediated endocytosis is the major pathway for selective internalization of plasma membrane receptors and their bound ligands. Both the coat assembly and the endocytic vesicle release depend on $PI(4,5)P_2$. The adaptor proteins AP2 and AP180, which are needed for clathrin assembly, both bind $PI(4,5)P_2$, as well as other clathrin adaptors such as epsin, Hip1/Hip1R and ARH/Dab. Some adaptors also bind to $PI(3,4,5)P_3$ (Wenk and de Camilli, 2004). Other proteins involved in such process are the large GTPase dynamin, which also binds $PI(4,5)P_2$ and AP2 and provides the machinery for the fission of the vesicle and the membrane (Schmid *et al.*, 1998), and the 5-phosphatase synaptojanin 1, which dephosphorylates $PI(4,5)P_2$ (and $PI(3,4,5)P_3$) and also binds AP2 and clathrin and interacts via its COOH-terminal targeting domain with several proteins with direct or indirect roles in endocytosis (Wenk and de Camilli, 2004).

Control of the Golgi Complex. The amount of $PI(4,5)P_2$ in the Golgi is nine fold lower than at the plasma membrane (Watt *et al.*, 2002). None of the PI(4)P5Ks have ever been visualised in this cellular compartment, but there is evidence of an associated PI(4)P5K activity associated which elevates $PI(4,5)P_2$ levels (Godi *et al.*, 1999; Jones D.H. *et al.*, 2000; Siddhanta *et al.*, 2000). There are three proposed functions for $PI(4,5)P_2$ in the Golgi complex that seem to be mediated by the control of spectrin and actin machineries: ER-to-Golgi transport (Godi *et al.*, 1998), formation and release of post-Golgi transport intermediates and a role in maintaining its structural integrity and function (Siddhanta *et al.*, 2000; Sweeney *et al.*, 2002). $PI(4,5)P_2$ might also be involved in the membrane fission process by

means of dynamin or PLD, producer of the fission-promoting lipid PA (Kozlov, 2001). What is clear is that the control of $PI(4,5)P_2$ levels in the Golgi are under the control of ARF proteins, which recruit PI4Kβ and PI(4)P5K to the Golgi membrane. Furthermore, ARF activates PI(4)P5Ks directly and indirectly through the activation of PLD, whose activity is $PI(4,5)P_2$-dependent and whose catalytic product, PA, is in turn an activator of PI(4)P5Ks. ARF itself can interact with $PI(4,5)P_2$ (Terui *et al.*, 1994; Randazzo *et al.*, 1997) and PA (Manifava *et al.*, 2001). Moreover, some ARF-GEFs and GAPs are capable of interacting with $PI(4,5)P_2$ and $PI(3,4,5)P_3$. All these enzymes acting together would provide an extremely powerful spatially and temporally controlled mechanism to increase $PI(4,5)P_2$ levels rapidly in selected membrane domains (de Matteis *et al.*, 2002).

Other functions. $PI(4,5)P_2$ regulates the direction of the axonal transport of mitochondria (de Vos *et al.*, 2003). $PI(4,5)P_2$ generation in the nucleus by both Type I and II PIPKs is linked to regulation of gene expression, pre-mRNA processing and mRNA export (Boronenkov *et al.*, 1998; Anderson *et al.*, 1999; York *et al.*, 1999).

$PI(3,4,5)P_3$

Phosphatidylinositol-3,4,5-trisphosphate is produced by phosphorylation of $PI(4,5)P_2$ by Class I PI3Ks (Heath *et al.*, 2003). $PI(3,4,5)P_3$ is transiently generated in response to stimuli and is mostly found at the plasma membrane (Cantley, 2002). The generation of this second messenger triggers a plethora of functions, most of which are summarised under the Class I PI3K functions section.

1.2 PHOSPHOINOSITIDE KINASES

There are three families of phosphoinositide kinases that phosphorylate the inositol ring in the three different positions that can be found in nature: 3-, 4- and 5-kinases.

Phosphoinositide 3-Kinases – PI3Ks

Phosphatidylinositol 3-kinases are responsible for the addition of a phosphate group to the D3 position of the inositol ring, generating PI(3)P, PI(3,4)P_2, PI(3,5)P_2 and PI(3,4,5)P_3. They all share a catalytic core with PI4Ks and the protein kinases target of rapamycin (TOR, also known as FRAP), ATM (the gene product mutated in the human disorder ataxia telangiectasia) and the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) (Wymann and Pirola, 1998; Vanhaesebroeck *et al.*, 2001). They are divided in three classes, as seen on Table 1.2.

Class I PI3Ks

There are four different isoforms divided in two subclasses, IA and IB. They are all heterodimers composed of a so-called p110 catalytic subunit and a regulatory subunit, p85, p55 or p50 in the case of IA and p101 in the case of IB.

Members. Class IA is composed of three different isoforms of the p110 catalytic subunit, α, β and δ. Class IB is composed of one sole isoform, p110γ, which was cloned in 1995 by Stoyanov *et al.* There are at least 7 regulatory subunits generated by expression and alternative splicing of three different genes, namely p85α (p55α and p50α), p85β, and p55γ for class IA enzymes (Wymann *et al.*, 2003). Class IB has one regulatory subunit, p101, with no similarity to any other known protein (Stephens *et al.*, 1997).

PI3K						
Class		Enzyme	MW	Regulatory subunits	Localisation	Substrate→Product
I	IA	p110α	123 (110)	p85α p55α p50α p85β p55γ	Cytosol Plasma Membrane	**$PI(4,5)P_2 \rightarrow PI(3,4,5)P_3$** *$PI \rightarrow PI(3)P$* *$PI(4)P \rightarrow PI(3,4)P_2$* $PI(5)P \rightarrow PI(3,5)P_2$ (only class IA)
		p110β	123 (119)			
		p110δ	119 (115)			
	IB	p110γ	120 (110)	p101		
II		PI3K-C2α	170/210	-	Clathrin-coated pits Plasma Membrane Microsomal Fractions Nucleus	**$PI \rightarrow PI(3)P$** *$PI(4)P \rightarrow PI(3,4)P_2$* $PI(4,5)P_2 \rightarrow PI(3,4,5)P_3$ (only in the presence of PS)
		PI3K-C2β	180			
		PI3K-C2γ	171			
III		PI3KIII	101	p150	Golgi and Endosomes	**$PI \rightarrow PI(3)P$**

Table 1.2 PI3K isoforms and properties.
The table shows the 3 different subfamilies of PI3Ks and the catalytic and regulatory subunits within each one. The molecular weight (MW) represents the predicted value in kDa of the different catalytic subunits, and in parentheses the observed value from SDS-PAGE migration studies. The first *substrate→product* pair in bold corresponds to the observed specificity *in vivo*; the rest are from *in vitro* studies with no proof of them occurring in living cells.

Substrates. Class IA enzymes are able to phosphorylate PI, PI(4)P, PI(5)P and $PI(4,5)P_2$ *in vitro* (Rameh *et al.*, 1997; Fruman *et al.*, 1998). p110γ phosphorylates the same substrates *in vitro* except for PI(5)P. However, when these kinases are stimulated *in vivo* by either surface receptors or Ras, only increases in $PI(3,4)P_2$ and $PI(3,4,5)P_3$ are registered, and $PI(3,4)P_2$ is thought to be the product of a 5-phosphatase acting on $PI(3,4,5)P_3$ rather than that of a direct phosphorylation reaction (Carpenter and Cantley, 1990).

Expression. p110α and p110β are ubiquitously expressed in mammalian tissues, where p110δ is predominantly found in leukocytes (Vanhaesebroeck *et al.*, 1997). p110γ is expressed highly in cells of haematopoietic origin and is present at low

concentrations in endothelia, smooth muscle cells and cardiomyocytes (Wymann *et al.*, 2003).

Localisation. They are thought to be mainly cytosolic under basal conditions, although they become membrane associated upon stimulation (Guillam *et al.*, 1999; Brock *et al.*, 2003).

Structure. All Class I PI3Ks catalytic subunits contain an internal C2 domain, a Ras-binding domain, a PI kinase accessory/helical domain and a catalytic kinase domain, as illustrated in Figure 1.4. Class IA enzymes also contain a p85-binding domain. The Class IB enzyme has an adaptor-binding site for p101 and two βγ

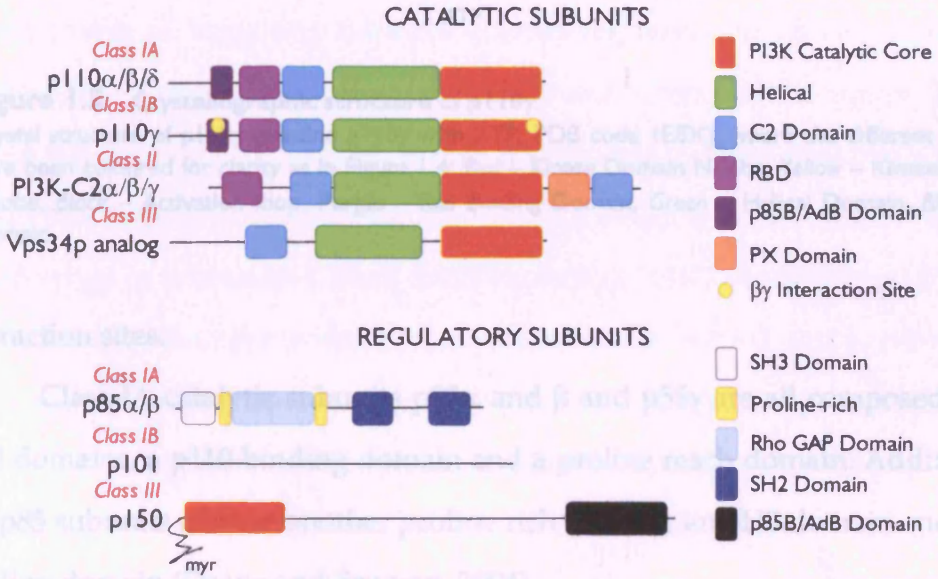


Figure 1.4 Diagrammatic representation of the domain structure of PI3K isozymes. The catalytic subunits of Class I enzymes are composed of p85-binding/adaptor-binding domain (Class IA/IB), Ras-binding domain (RBD), PKC homology domain 2 (C2), PI3K accessory domain and catalytic core domain which interacts directly with ATP, $PI(4,5)P_2$ and wortmannin. p110γ has two additional βγ interaction sites. Class II enzymes lack an AdB site but possess additional phox (PX) and C2 domains. Class III enzymes share the PI3K catalytic and accessory domains, as well as one C2 domain. The Class IA regulatory subunits possess one Src homology 3 (SH3) and two Src homology 2 (SH2) domains, in addition to a Rho-GAP domain and two proline rich regions. The Class IB regulatory subunit, p101, has no resemblance to any other know protein structure. The Class III regulatory subunit has a PI3K catalytic core, a WD-40 domain and an N-terminal myristate.

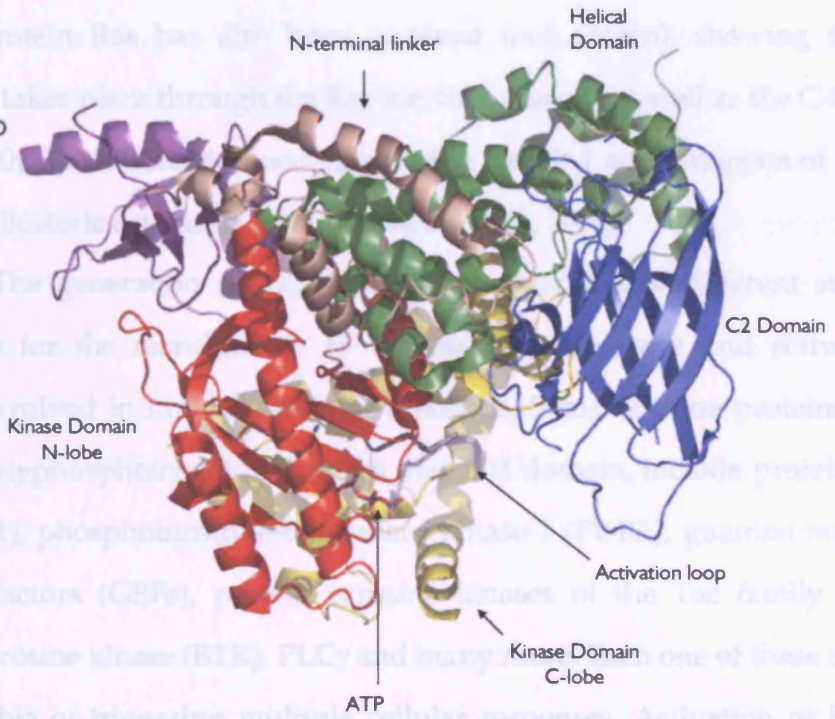


Figure 1.5 **Crystallographic structure of p110γ.**
Crystal structure of p110γ (porcine p110γ with ATP; PDB code 1E8X), where the different domains have been coloured for clarity as in Figure 1.4: *Red* – Kinase Domain N-lobe, *Yellow* – Kinase Domain C-lobe, *Black* – Activation loop, Purple – Ras Binding Domain, *Green* – Helical Domain, *Blue* – C2 Domain.

interaction sites.

Class IA catalytic subunits p85α and β and p55γ are all composed of two SH2 domains, a p110-binding domain and a proline reach domain. Additionally, the p85 subunits contain another proline rich domain, an SH3 domain and a Rac-binding domain (Deane and Fruman, 2004).

The structure of p110γ has been resolved with the exclusion of the N-terminal regions that bind the adaptor protein p101 as seen in Figure 1.5. Its structure is very similar to that of protein kinases, although the nucleotide-binding loop is different in that it lacks the consensus glycine-rich GxGxxG sequence motif (Walker *et al.*, 1999). The structure of p110γ together with the

small G protein Ras has also been resolved (not shown), showing that this interaction takes place through the Ras-binding domain as well as the C-terminal lobe of p110γ. The interaction occurs with the switch 1 and 2 regions of Ras and causes an allosteric activation of PI3K (Pacold *et al.*, 2000).

Function. The generation of $PI(3,4,5)P_3$ upon reception of different stimuli is responsible for the recruitment to the plasma membrane and activation of enzymes involved in multiple cellular functions. Some of these proteins, which bind the polyphosphoinositide through their PH domain, include protein kinase B (PKB/Akt), phosphoinositide-dependent kinase 1 (PDK1), guanine nucleotide exchange factors (GEFs), protein tyrosine kinases of the Tec family such as Bruton's tyrosine kinase (BTK), PLCγ and many more. Each one of these enzymes is responsible of triggering multiple cellular responses. Activation of PKB/Akt implies binding to this polyphosphoinositide and subsequent phosphorylation of the enzyme by PDK1 on Thr 308. Maximal activation requires phosphorylation of Ser473 by an activity called PDK2, the nature of which is controversial. PKB has a broad range of substrates (Brazil and Hemmings, 2001). Activation of PKB and PDK results in the counteraction of apoptosis so promoting **cell survival**. They also regulate the activity of the nutrient sensor TOR, which together with PDK1 promote transcription, translation, and glucose uptake, all necessary for **cell growth** prior to cell division (Wymann *et al.*, 2003). **Proliferation** is also controlled by PKB-dependent inactivation of glycogen synthase kinase 3β (GSK-3β), which leads to a rise in cyclin D1 and entry into the cell cycle. **Motility** is also dependent on the recruitment of GEFs for different small G proteins onto the plasma membrane by binding to $PI(3,4,5)P_3$; these in turn recruit the GTPases, such as Rac and Cdc42, which are involved in the rearrangement of the actin cytoskeleton and migration. All these signals provide the mechanisms that

render cells tumourigenic and malignant, making their understanding a key factor in the fight against **cancer** (Vivanco and Sawyers, 2002). $PI(3,4,5)P_3$ generation is also crucial in immunological responses; translocation of Tec family kinases such as Btk or phospholipases like PLCγ, is instrumental in increasing the generation of DAG. In turn, DAG activates PKCs, which along with a rise in intracellular calcium concentration due to IP_3, together induce **clonal-selection** processes in B and T cells and also mediate **degranulation** in mast cells and other granulocytes (Fruman and Cantley, 2002). This is a clear demonstration of the type of signalling crosstalk that inositol lipid metabolism controls in mammalian cells.

Each p110 isoform has specific unique functions within the cells. For instance, p110γ is essential in neutrophil and lymphocyte function, as deduced from knock-out approaches in mice (Cantrell, 2001). Mice lacking p85α are not viable but p85α-lacking B cells are functionally deficient whereas T cells seem completely normal. In macrophages, p110α is required for mitogenic responses triggered by colony-stimulating factor 1 (CFS-1), whereas p110β and δ are necessary for CSF-1 induced modulation of actin dynamics (Vanhaesebroeck *et al.*, 1999).

PI 3-kinase also exhibits protein kinase activity which is involved in activation of the MAPK cascade (Bondeva et al., 1998).

Regulation. The activation of Class IA PI3Ks is dependent on the recruitment of the catalytic subunit p110 by the adaptor subunit p85. The adaptor subunit is in turn recruited by binding of its SH2 domains onto tyrosine-phosphorylated residues with consensus pYxxM, downstream of receptor tyrosine kinases or RTKs, their substrates or associated adaptor proteins. This recruitment gives p110 access to its lipid substrate (Gillham *et al.*, 1999); and the contact of p85 with

the tyrosine-phosphorylated receptor induces a conformational change of p85 that results in dishinibition of p110 enzymatic activity (Yu *et al.*, 1999).

On the other hand, the sole Class IB enzymatic complex, p101/p110γ, is recruited to the plasma membrane through interaction with the βγ-subunits of heterotrimeric G proteins. These subunits are dissociated from the heterotrimer upon activation of G protein-coupled receptors. p101 is thought to be responsible for the recruitment of p110γ upon release of βγ-subunits (Brock *et al.*, 2003). Interaction with GTP-bound Ras is proposed to induce conformational changes and membrane reorientation of the lipid kinase in relation to its substrate, further increasing its activity. Although p101 and Ras contribute to PI3Kγ activation, the sequence of actions and the quantitative contribution of PI3Kγ-interacting proteins in physiological processes need further elucidation. PI3Kγ–βγ–p101–Ras interaction leads to massive production of PtdIns(3,4,5)P_3 (Wymann *et al.*, 2003).

Class II PI3Ks

Members. So far three members of this subfamily of PI3Ks have been identified, PI3K-C2 (Class II PI3K) α, β and γ.

Substrates. *In vitro* substrates include PI and PI(4)P, and PI(4,5)P_2 exclusively in the presence of PS (Domin *et al.*, 1997).

Expression. The α and β isoforms are ubiquitously expressed; PI3K-C2γ is found in hepatocytes, and a N-terminal truncated form was found in lung and a certain haematopoietic cell line (Misawa *et al.*, 1998).

Localisation. Class II enzymes are mostly associated with the plasma membrane and with the low-density microsomal fraction (Vanhaesebroeck *et al.*, 1999). PI3K-C2α has been reported to localise in clathrin-coated structures at the plasma membrane and TGN (Domin *et al.*, 2000; Gaidarov *et al.*, 2001) and also in

the nucleus together with PI3K-C2β (Didichenko and Thelen, 2001; Sindic *et al.*, 2001).

Structure. Class II PI3Ks are large, 170-210 kDa, monomeric enzymes containing an internal C2 domain, Ras-binding domain, PI kinase homology domain and kinase domain, which are highly similar (45-50%) to those in Class IB (MacDougall *et al.*, 1995). These PI3Ks also have another C2 domain and PX domain in their N-terminus (Fruman *et al.*, 1998). This C2 domain can bind *in vitro* to phospholipids in a Ca^{2+}-independent manner. The N-terminal regions contain a Ras binding domain, although there is no evidence of binding to this small G protein (MacDougall *et al.*, 1995).

Function. Despite much investigation this is not clear.

Regulation. Their activity and location are thought to be regulated by extracellular stimuli; indeed, increases in lipid kinase activity from PI3K immunoprecipitates have been seen to be induced upon stimulation with insulin, epidermal growth factor, platelet-derived growth factor, integrin binding and chemokines (Vanhaesebroeck *et al.*, 2001).

Class III PI3Ks

Members. Only one isoform of this class has been found, present from yeast to mammals. It is the homolog of the yeast Vps34p (Odorizzi *et al.*, 2000).

Substrates. The only substrate it can phosphorylate is PI to generate PI(3)P *in vivo*.

Localisation. It is localised to endocytic vesicles (Stephens *et al.*, 2002).

Structure. Like class II enzymes, they contain the internal C2 domain, PI3K catalytic core and helical domain.

Function. This kinase is involved in protein and vesicle trafficking, as well as trafficking processes unique to immune cells, especially phagocytosis (Stephens *et al.*, 2002).

Regulation. Both yeast and mammalian isoforms are regulated by interaction with a serine/threonine kinase and Vps15p or p150 respectively, which are N-terminal myristoylated. This serves as a mechanism for targeting the lipid kinase to membranes (Panaretou *et al.*, 1997). The constant levels of PI(3)P in cells suggest for a control mechanism of this class of PI3Ks independent of cellular stimulation, being most probably constitutively activated (Vanhaesebroeck *et al.*, 2001).

Phosphoinositide 4-Kinases - PI4Ks

Phosphatidylinositol 4-kinases add a phosphate group to the D4 position of the inositol ring, directly generating PI(4)P. There are several PI4K isoforms (Table 1.3) whose activity must be strictly regulated, as other phosphoinositide kinases depend on the availability of the D4-phospho-stereoisomer to generate other PIs, mainly PI(4,5)P_2 and PI(3,4,5)P_3 (Fruman *et al.*, 1998).

PI4K						
	Enzyme	MW	Yeast ortholog	Localisation	Substrate→ Product	Biochemical Properties
II	PI4K α	55	PIK2 (LSB6)	Endosomes	PI→PI(4)P	- Inhibited by Adenosine and Ca^{2+} - Low Km for ATP and PI - Wortmannin insensitive
	PI4K β			P.Mb./Golgi/ER		
III	PI4K α	230	STT4	ER	PI→PI(4)P	- Insensitive to Adenosine and Ca^{2+} - High Km for ATP and PI - Sensitive to wortmannin IC_{50} 50-100nM LY294002 IC_{50} 50-100μM
	PI4K β	93	PIK1	Golgi		

Table 1.3 **PI4K isoforms and properties.**
The table shows the 2 different subfamilies of PI4Ks, their molecular weight (MW) in kDa, cellular localisation, phosphorylating-substrate and product and biochemical properties.

Type II PI4Kα

Type II PI4Ks are the major contributors to cellular PI(4)P in response to extracellular signal transduction (Fruman *et al.*, 1998). They are membrane associated enzymes inhibited by adenosine (K_i < 50 μM) and Ca^{2+}, that exhibit lower Km values for ATP and PI and which are insensitive to wortmannin and LY294002 (Balla T., 1998; Fruman *et al.*, 1998). The single yeast homolog of mammalian PI4KIIs is PIK2 (LSB6) (Shelton *et al.*, 2003).

Type II PI4Kα is membrane associated through a palmitoylation site in the centre of the 55 kDa protein (Barylko *et al.*, 2001). It is located at the Golgi and at synaptic membranes (Guo *et al.*, 2003; Wang *et al.*, 2003). The enzymes product, PI(4)P, as well as serving as a precursor of $PI(4,5)P_2$, is involved in membrane traffic from the *trans*-Golgi network as it is required for membrane transport to the plasma membrane (Wang *et al.*, 2003), and in the exo-endocytic cycle of synaptic vesicles (Guo *et al.*, 2003). Some of this activity is thought to arise because AP1-containing clathrin coats recognise and bind PI(4)P.

Type II PI4Kβ

Type II PI4Kβ has a high degree of similarity with type IIα in its C-terminal catalytic domain, but greater divergence in its N-terminus (Balla A. *et al.*, 2002). This isoform is cytosolic and is recruited primarily to the plasma membrane and activated by Rac1-GTP, although it can also be found at the Golgi and ER. PI4KIIβ is activated strongly by membrane association to stimulate $PI(4,5)P_2$ synthesis at the plasma membrane (Wei *et al.*, 2002).

Type III PI4Kα/STT4

In yeast, mutants of STT4 do not affect the secretory pathway but disrupt actin remodelling. The mammalian isozymes contain a PH domain for

membrane binding (Wong and Cantley, 1994) and several domains for protein-protein interaction and nucleic acid binding domains. Potential phosphorylation sites in PI4KIIIα suggest regulatory function of PKA, PKC, caseine kinase II and protein tyrosine kinases (Gehrmann *et al.*, 1999).

Type III PI4Kβ/PIK1

In yeast, PI(4)P generated by this inositide kinase has been proposed to be involved in secretory pathway trafficking (Balla T. *et al.*, 1998) and in Golgi to plasma membrane transport (Walch-Solimena and Novick, 1999). PIK1 is not sensitive to wortmannin. Both mammalian and yeast isozymes behave as soluble proteins; hence, their membrane association must be tightly regulated. Indeed, PI4KIIIβ has been localised to the Golgi where is recruited by the GTPase ARF (Godi *et al.*, 1999).

Phosphatidylinositol Phosphate Kinases – PIPKs

Three different subfamilies of phosphatidylinositol *x*-phosphate *y*-kinases have been reported and they have been grouped by the similarity of their conserved kinase domains. Initially, all the kinases in this group were thought to use PI(4)P as a substrate to generate the important second messenger and promoter of second messengers $PI(4,5)P_2$. However, later studies have demonstrated that even though they share homology in their catalytic domains, they use different substrates to generate PIP_2 and they have diverse localisation and functions within cells.

PIPK						
	Enzyme	MW	Yeast	Localisation	Substrate→Product	Functions
I	PI(4)P5K α PI(4)P5K β PI(4)P5K γ	56/61/62 (68) 61/57 (68) 69/72/75 (87/90/-)	MSS4	Plasma Mb. Nucleus Golgi Focal adhesions	**PI(4)P→PI(4,5)P_2** *PI→PI(5)P* *PI(3)P→PI(3,4)P_2* *PI(3)P→PI(3,5)P_2* *PI(3)P→PI(3,4,5)P_3* PI(3,4)P→PI(3,4,5)P_3	- Actin reorganisation - Secretion - Endocytosis: receptor-mediated and trafficking - Apoptosis - Ion channel regulation
II	PI(5)P4K α PI(5)P4K β PI(5)P4K γ	47 (53)	-	Cytosol Nucleus ER Actin cytoskeleton	**PI(5)P→PI(4,5)P_2** *PI(3)P→PI(3,4)P_2* *PI(3)P→PI(3,4,5)P_3*	- Uncertain
III	PIKfyve	235	FAB1	Late endosomes	**PI(3)P→PI(3,5)P_2** *PI→PI(5)P*	- Membrane trafficking

Table 1.4 PIPK isoforms and properties.
The table shows the three types of PIPKs with the different isoforms within each group. The molecular weight (MW) represents the predicted value in kDa of the different existent splice variants, and in parentheses the observed value from SDS-PAGE migration studies. The first substrate→product pair in bold corresponds to the observed specificity *in vivo*; the rest are from *in vitro* studies with no proof of them occurring in cells.

Type I PI(4)P5Ks – Genuine PI(4)P5Ks

These kinases were the ones that originally gave name to the whole family.

Substrates. Out of the three subfamilies known to date, Type I PI(4)P5Ks are the only ones that genuinely use PI(4)P as a substrate to produce PI(4,5)P_2 *in vivo*. However, they seem to be more promiscuous *in vitro*, where at least 5 different phosphorylation reactions using different substrates have been reported as seen in Table 1.4 (Hinchliffe *et al.*, 1998a). The only other one of those reactions that has been detected *in vivo* is the phosphorylation of PI(3,4)P_2 to generate PI(3,4,5)P_3 by Type I PI(4)P5K in cells exposed to oxidative stress (Halstead *et al.*, 2001).

Members. The subfamily is composed of three different isoforms and several splice variants of each one of them. PI(4)P5Kα and β were first cloned by Ishihara *et al.* (1996) from a cDNA library of the murine pancreatic β-cell line MIN6, and later the same group cloned the γ isoform (1998) using the same cDNA library. There are three splice variants of the human α isoform (which corresponds to murine PI(4)P5Kβ due to a long-standing divergence in nomenclature that has never been corrected), namely α_1, α_2 and α_3, all with slightly different molecular weights, and two of the human β isoform (murine PI(4)P5Kα), β_1 and β_2 (Loijens *et* al., 1996b; Carvajal *et al.*, 1996). Three different splice variants of PI(4)P5Kγ (also murine PI(4)P5Kγ) have been reported so far: PI(4)P5Kγ661, PI(4)P5Kγ635 and PI(4)P5Kγ688 (Ishihara *et al.*, 1998; Giudici *et al.*, 2004). Recently, a PI(4)P5K homolog with no apparent kinase activity has been identified and named PIPKH. It heterodimerises with other PI(4)P5Ks, acting as a scaffold to localise and regulate PI(4)P5Ks and the synthesis of $PI(3,4,5)P_3$; its presence provokes 8-fold increases in total $PI(3,4,5)P_3$ without significantly altering $PI(4,5)P_2$ levels (Chang *et al.*, 2004).

Expression. Type I PI(4)P5Ks are found in plants, yeast and mammalian cells. PI(4)P5Kα is mainly expressed in skeletal muscle, and it is also present in heart, placenta, kidney and pancreas. Low levels were reported in brain, liver and lung. PI(4)P5Kβ is also present in most tissues examined except for liver. It is especially abundant in heart and moderately expressed in brain and pancreas (Loijens and Anderson, 1996a). PI(4)P5Kγ661 is widely expressed, being most preferentially found in kidney, lung and, in higher amounts, in brain (Ishihara *et al.*, 1998; Wenk *et al.*, 2001). PI(4)P5Kγ635 is less broadly distributed and is hardly detectable in brain and testis, whereas the PI(4)P5Kγ688 transcript is exclusively

detected in brain (Giudici *et al.*, 2004). PIPKH is expressed at relatively high levels in brain and testis (Chang *et al.*, 2004).

Localisation. Type I PI(4)P5Ks are principally localised at the plasma membrane, in the Golgi, in the nucleus and at focal adhesions. Moreover, their subcellular distribution seems to vary depending on the isoform. Endogenous human PI(4)P5Kα (mouse β) can be found in the nucleus where it associates with nuclear speckles (Boronenkov *et al.*, 1998), in membrane ruffles, dependent upon association with Rac and/or ARF6 (Honda *et al.*, 1999; Doughman *et al.*, 2003b), and on the plasma membrane during phagocytosis (Botelho *et al.*, 2000; Coppolino *et al.*, 2002). Human PI(4)P5Kβ (mouse α) is present in a vesicular perinuclear region (Doughman *et al.*, 2003a). PI(4)P5Kγ661 is targeted to focal adhesions by its 26-amino acid C-terminus. PI(4)P5Kγ635 is also located at the plasma membrane but not in these specialised adherence domains, for it lacks the extreme 26 amino acids of its N-terminus which are required for such interaction (di Paolo *et al.*, 2002; Ling *et al.*, 2002).

Structure. PI3Kinases and PI4Kinases share some statistically significant sequence similarity, but PIPKinases do not have any strong sequence homology with the other two groups of PI kinases (Heath *et al.*, 2003). Nevertheless, the crystallisation of Type II PI(4)P5Kβ (or PI(5)P4Kβ) and mutation analysis have revealed that they share some structural features with other kinases, such as activation loops and putative nucleotide binding sites with conserved catalytic residues that bind ATP and Mg^{2+}; all this implies that there is a common phosphotransfer mechanism and that structurally, the phosphoinositide kinases all belong to a superfamily (Ishihara *et al.*, 1998; Rao *et al.*, 1998; Kunz *et al.*, 2000, 2002). The activation loop of Type I and II PIPKs has a dual role in substrate specificity and in promoting membrane association. Using site-directed

mutagenesis studies, Kunz *et al.* (2002) demonstrated that a single amino acid change was capable of swapping the stereo-specific substrate recognition of the type I and II PIPKs, as well as their subcellular membrane targeting. This means they can swap between utilising PI(4)P or PI(5)P to generate $PI(4,5)P_2$. Recently, Arioka *et al* (2004) have shown how the presence of dibasic residues at the C-terminal end of the kinase homology domain, particularly the dibasic Arg-Lys sequence, is necessary and sufficient for the proper localization and cellular function of PIP5Ks. These residues are conserved among mammalian as well as invertebrate PIP5K family members, but not in the type II PIPKs that are not targeted to the plasma membrane.

The kinase domain of PI(4)P5Ks is comprised of around 400 amino acids located centrally and with 70-80% similarity amongst the three proteins, 40% identity

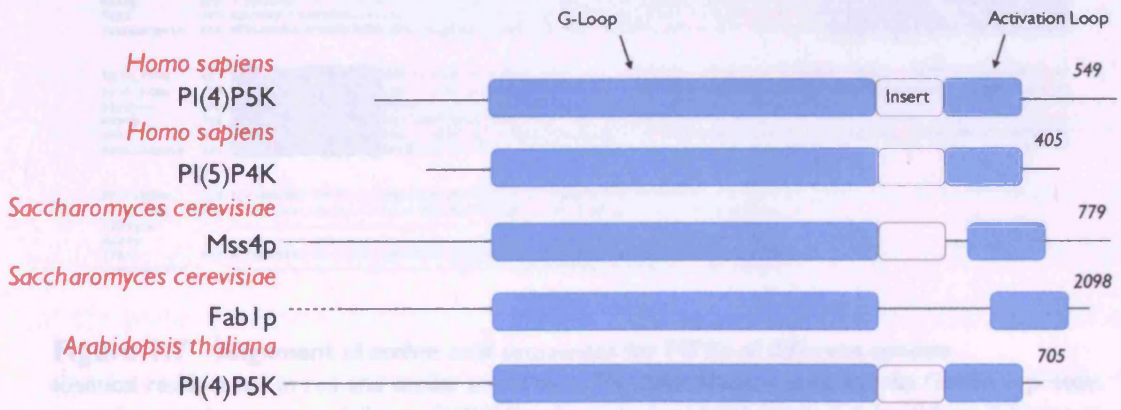


Figure 1.6 **Diagrammatic representation of the kinase homology domain of PIPKs of *Homo sapiens*, *Saccharomyces cerevisiae* and *Arabidopsis thaliana*.**
The kinase homology domain of PIPKs is represented in blue. The kinase domain, except in Fab1p homologs, is separated by an insert region, represented in white.. The activation loop is part of the second half of the sequence of the homology domain. Although statistically there is no great global similarity with other kinases, there are local similarities in the conserved sequence motifs characteristic of protein (and lipid) kinases . For example, glycine-rich motif (GXSGS) G-Loop in the homologs resembles the same phosphate-binding loop of protein kinases and other ATP-binding proteins.

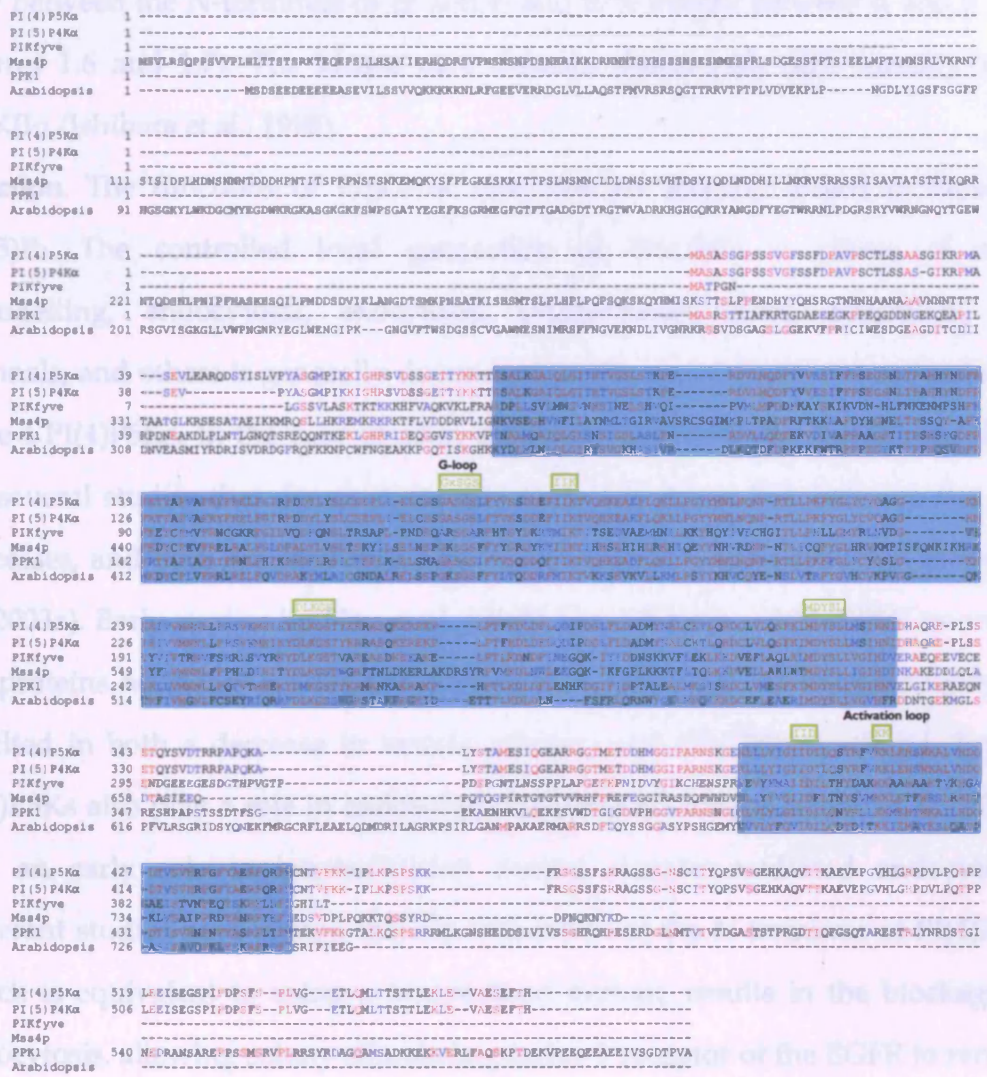


Figure 1.7 Alignment of amino acid sequences for PIPKs of different species.
Identical residues are in red and similar are in blue. The abbreviations used and the GenBank protein accession numbers are as follows. *PI(4)P5Kα*: Type I phosphatidylinositol 4-phosphate 5-kinase α *Homo sapiens* (AAC50910); *PI(5)P4Kα*: Type II phosphatidylinositol 4-phosphate 5-kinase α *Homo sapiens* (AAC50910) PIKfyve: Type III phosphatidylinositol 4-phosphate 5-kinase *Homo sapiens* (P53807); Mss4p: Mss4p *Saccharomyces cerevisiae* (NP 010494); PPK1: Pip Kinase protein I *Caenorhabditis elegans* (AAB54130); Arabidopsis: phosphatidylinositol 4-phosphate 5-kinase *Arabidopsis thaliana* (AAB82658). The two fragments of the Kinase Homology domain are coloured in blue, and the insert lies in between the two sequences. Fragments in green correspond to regions of identity (structural regions are labelled). Alignment was generated by **ClustalW** (www.ebi.ac.uk/clustalw) and the printing of the multiple alignment output was performed by **Boxshade** (www.ch.embnet.org/software/ BOX_form.html). Domain determination was generated using **CDD** (Marchler-Bauer *et al.*, 2003).

only between the N-terminus of Iβ and Iγ and 67% overall between α and β (see Figures 1.6 and 1.7). The kinase core domain shares only 40% identity with PIPKIIα (Ishihara *et al.*, 1998).

Function. The functions of PI(4)P5K enzymes are directly linked to those of $P(4,5)P_2$. The controlled local generation of $PI(4,5)P_2$ in places of actin remodelling, endocytosis, exocytosis, phagocytosis, focal adhesions, ion channels, and others is generally dependent on the recruitment and activation of Type I PI(4)P5Ks. In the case of actin dynamics and vesicular trafficking, there are several studies that demonstrate that there is a direct link between the two processes, and both are modulated by Type I PI(4)P5K isoforms (Doughman et al., 2003a). Early studies by Hay et al. (1995) identified Type I PI(4)P5K as one of the proteins necessary for regulated exocytosis, and thus, its immunodepletion resulted in both a decrease in vesicle priming and $PI(4,5)P_2$ synthesis. Type I PI(4)P5Ks also play a role in endosomal vesicle trafficking (Galiano et al., 2002), and an early role in internalisation during receptor-mediated endocytosis. Different studies have shown that the truncation of the N-terminus of PI(4)P5K, which is equivalent to using a kinase-dead mutant, results in the blockage of endocytosis, allowing colony stimulating factor-1 receptor or the EGFR to remain at the plasma membrane. This occurs by blockade of the recruitment of clathrin light chain and dynamin to the plasma membrane (Cochet et al., 1991; Davis et al., 1997; Barbieri et al., 2001). PI(4)P5K is also involved in neurite remodelling in response to a number of diverse stimuli (van Horck et al., 2002; Yamazaki et al., 2002) and regulation of axonal elongation and branching downstream of ARF6 and ARNO (Hernández-Deviez et al., 2004), amongst several other functions.

Regulation. The activity of Type I PI(4)P5Ks can be regulated by different factors, summarised in Figure 1.8.

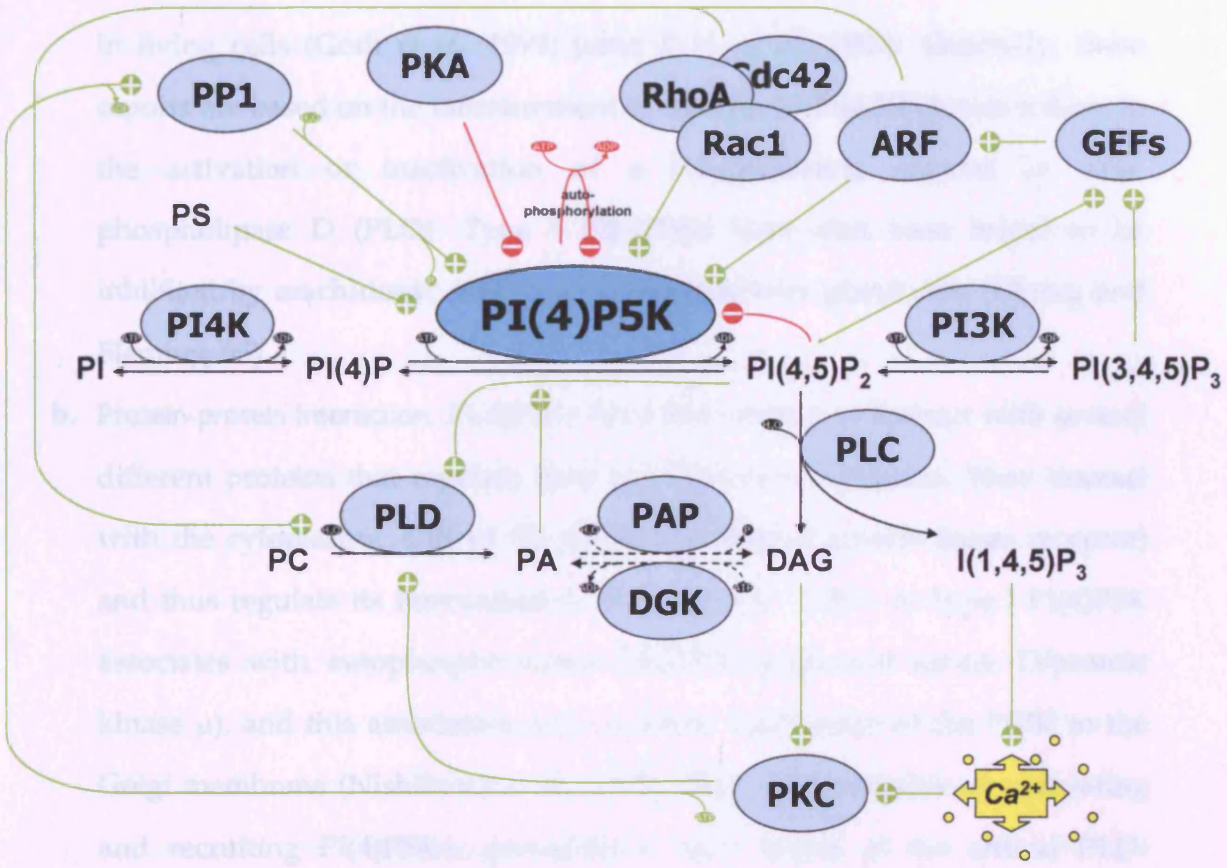


Figure 1.8 **Regulation of PI(4)P5K enzymes by lipids, protein-protein interaction and de/phosphorylation mechanisms.**
Phosphoinositide phosphatases have been excluded for simplicity.

a. Lipids. Type I PI(4)P5Ks are activated by **PS** (Cochet and Chambaz, 1986) and **PA** *in vitro* (Moritz *et al.*, 1992; Jenkins *et al.*, 1994; Ishihara *et al.*, 1996, 1998) and inhibited by its own product **PI(4,5)P$_2$** (Moritz *et al.*, 1992). The extent of the activation, which varies between 2-fold and 20-fold, is extremely dependent on the composition of the lipid vesicles and the origin of the lipid kinase; so much indeed that in the presence of **PC** these effects can be completely abolished (Jones D.H. *et al.*, 2000). However, no direct activation *in vivo* has ever been demonstrated. There are contradictory reports that give evidence of this regulation (Arneson *et al.*, 1999; Jones D.R. *et al.*, 2000; O'Luanaigh *et al.*, 2002; Skippen *et al.*, 2002) or absence of such an activation

in living cells (Godi *et al.*, 1999; Jones D.H. *et al.*, 2000). Generally, these reports are based on the measurement of changes in $PI(4,5)P_2$ levels subject to the activation or inactivation of a PA-generating enzyme in cells, phospholipase D (PLD). Type I PI(4)P5Ks have also been found to be inhibited by **arachidonic acid** in rat submandibular gland cells (Chung and Fleming, 95).

b. Protein-protein Interaction. PI(4)P5Ks have been shown to interact with several different proteins that regulate their localisation or activation. They interact with the cytoplasmic tails of the **EGFR** (epidermal growth factor receptor) and thus regulate its internalisation (Cochet *et al.*, 1991). A Type I PI(4)P5K associates with autophosphorylated **PKD/PKCμ** (protein kinase D/protein kinase μ), and this association may facilitate localisation of the PIPK to the Golgi membrane (Nishikawa *et al.*, 1998). **PLD** is also capable of associating and recruiting PI(4)P5Kα, providing a local source of the critical PLD-activator $PI(4,5)P_2$ (Divecha *et al.*, 2000). Another lipid kinase which has been reported to associate with PI(4)P5K is **DGKζ** (diacylglycerol kinase ζ). They both co-localise with actin in lamellipodial protrusions, supporting the idea that a DGK can generate PA in order to locally activate PI(4)P5K and thus increase $PI(4,5)P_2$ levels, which in turn control actin polymerisation (Luo *et al.*, 2004). PI(4)P5Kγ661 interacts with and becomes strongly activated by **talin**, a principal component of focal adhesion plaques that is also present in neurons, suggesting a direct involvement of the PI(4)P5K in cell adhesion and synaptic junctions (di Paolo *et al.*, 2002; Ling *et al.*, 2002). **BTK** (Bruton's tyrosine kinase), a member of the Tec family of cytoplasmic protein tyrosine kinases, associates with PI(4)P5Ks upon B cell receptor activation, bringing PI(4)P5K to the plasma membrane as a means of generating local $PI(4,5)P_2$ synthesis.

This protein-protein interaction allows BTK to stimulate the production of the substrate required by both its upstream activator, PI3K, and its downstream target, PLCγ2 (Saito *et al.*, 2003).

As Type II PIPKs do, Type I PI(4)P5Ks also form **dimers**, and possibly oligomeric protein complexes thanks to the presence of two independent dimerisation domains found within murine Type I PI(4)P5Kβ (Galiano *et al.*, 2002).

Finally, a crucial method of regulation of Type I PI(4)P5Ks is interaction with small G proteins. This type of regulation will be described separately in section **d**.

c. **Phosphorylation.** There is ample evidence of regulation of Type I PI(4)P5Ks by phosphorylation by different protein kinases, which overall has an inhibitory effect on their activity. In *Schizosaccharomyces pombe*, a Type I-like PIPK was shown to be phosphorylated and inhibited *in vitro* by the **casein kinase I** homologue Cki1 and overexpression of this kinase reduces the cellular levels of $PI(4,5)P_2$ (Vancurova *et al.*, 1999). **Autophosphorylation** of Type I PIPKs has been demonstrated to be another way of downregulating their activity. Itoh *et al.* (2000) showed how in the presence of ATP and Mg^{2+} (and specially enhanced by the presence of PI), PI(4)P5Kβ autophosphorylates on serine residues and subsequent phosphatase treatment is able to increase its 5-kinase activity. LPA stimulation of Type I PI(4)P5Ks has been shown to directly correlate with **PP1**-mediated dephosphorylation of the enzyme upon PKC activation (Park *et al.*, 2001). These authors also suggested that **PKA** might be responsible for the initial phosphorylation event, as treatment of 3T3 cells with the PKA inhibitor, H89, but not with other protein kinase inhibitors, was able to reduce the resting phosphorylation of P(4)P5Kβ. Wenk

et al. (2001) have also reported that the major neuronal PI(4)P5Kγ isoform undergoes dephosphorylation upon depolarisation of synaptosomes, which induces an increase in phosphoinositide turnover, although no correlating change in activity has been shown. PI(4)P5Kγ661 also becomes tyrosine phosphorylated by **FAK** (focal adhesion associated kinase) signalling, increasing both its activity and its association with talin, although it is not clear whether the increase in activity is due directly to the phosphorylation or indirectly to the increased association with talin (Ling *et al.*, 2002).

d. **Small G proteins.** Members of the Rho family of small G proteins, particularly Rho and Rac, and also members of the ARF family are involved in actin dynamics and vesicle trafficking, and so is $PI(4,5)P_2$, by modulating signal transduction pathways and the activity of multiple actin-binding proteins. Therefore, a correlation between action of these small G proteins and PIPK activity exists and evidence shows that their direct interaction modulates the intracellular targeting of PI(4)P5Ks as a way of controlling the spatial production of $PI(4,5)P_2$.

Rho, a small G protein involved in the assembly of actin stress fibres and focal adhesion (Ridley and Hall, 1992), has been shown to interact with all three isoforms of PI(4)P5K in a GTP-independent manner and promote $PI(4,5)P_2$ synthesis in cells (Ren *et al.*, 1996; Weernink *et al.*, 2004). The activation of PI(4)P5K by Rho seems to be dependent upon the Rho effector ROCK (Rho kinase), independently of phosphorylation (Oude Weernink *et al.*, 2000; van Horck *et al.*, 2002; Yamazaki *et al.*, 2002; Weernink *et al.*, 2004) and it seems conceivable that the effect of Rho on PI(4)P5K rather than directly activating the kinase is its recruitment to specific cellular compartments (Chatah *et al.*, 2001; Weernink *et al.*, 2004). Both Rho and ROCK

are responsible for the recruitment of the lipid kinase and regulation of the assembly of the actin cytoskeleton upon thrombin-stimulation in platelets (Yang *et al.*, 2004). In contrast, there is one report in the literature where the activation of PI(4)P5K by Rho is independent of ROCK (Matsui *et al.*, 1999).

Rac, mediator of growth factor-induced membrane ruffling and lamellipodia formation, has also been found to interact with all PI(4)P5K isoforms in a GTP-independent manner and promote $PI(4,5)P_2$ synthesis in cells (Tolias *et al.*, 1995, 1998a, 1998b, 2000; Weernink *et al.*, 2004). Moreover, Tolias and colleagues found that PI(4)P5K associated with the small GTPase in conjunction with a DGK, possibly the ζ isoform, forming a lipid kinase signalling complex. The apparent independence of the activation state of both Rac and Rho (GTP- or GDP-bound) during association with PI(4)P5K, points at a binding-region outside the switch regions that normally undergo conformational rearrangements upon nucleotide changes. Indeed, in the case of Rac this region is located in the C-terminus and is necessary and sufficient for binding to both lipid kinases (Tolias *et al.*, 1998b). As with Rho, it seems likely that the effect of Rac on the lipid kinase has more to do with localisation than with activation (Tolias *et al.*, 2000b; Weernink *et al.*, 2004).

Cdc42 has also been reported to stimulate the production of $PI(4,5)P_2$ in cells, but no direct physical interaction takes place between the two proteins (Weernik *et al.*, 2004).

ARF proteins are another family of small G proteins that interact and activate PI(4)P5Ks. As these GTPases are the main focus of this thesis, an in depth revision of their characteristics and properties will be carried out on the following section. Regarding their interaction with Type I PI(4)P5Ks, there was evidence of the influence of ARF proteins in the generation of $PI(4,5)P_2$ in

cells before the finding that ARF specifically stimulated PI(4)P5K activity (Fensome *et al.*, 1996; Martin *et al.*, 1996; Godi *et al.*, 1999). In 1999, Honda *et al.* demonstrated that ARF was capable of activating PI(4)P5Kα in the presence of PA, observation that has later been corrected, for the presence of the anionic lipid is not required (Jones D.H. *et al.*, 2000). In some cases, the cellular increase in $PI(4,5)P_2$ synthesis seems to occur by the coordinated activation of both PI(4)P5K and PLD (Honda *et al.*, 1999; Skippen *et al.*, 2002; O'Luanaigh *et al.*, 2002), which is also activated by ARF proteins, generating a positive feedback loop since each enzymes product is an activator of the other one (Jenkins *et al.*, 1994; Powner and Wakelam, 2002). However, in other examples it has been shown that the PA generated by PLD does not influence the activation of PI(4)P5K by ARF (Jones D.H. *et al.*, Godi *et al.*, 1999; Lawrence *et al.*, 2003). Hence, this still remains a contentious subject that has not been resolved. Moreover, Type I PI(4)P5K has been proven to function and be recruited to plasma or endosomal membranes downstream of ARF6 (Honda *et al.*, 1999; Brown *et al.*, 2001; Krauss *et al.*, 2003; Aikawa and Martin, 2003) or of an unidentified ARF to Golgi membranes (Godi *et al.*, 1999). In the case of membrane ruffling induced by EGF stimulation, the generation of $PI(4,5)P_2$ at the plasma membrane is essential and ARF6 seems to be responsible for the co-translocation of PI(4)P5Kβ and PLD2 both by Rac1-dependent and independent mechanisms; nevertheless, a Rac1-dependent ARF6-independent pathway also seems to be required for the membrane ruffling to occur (Honda *et al.*, 1999). In terms of the association of ARF and PI(4)P5Ks, two contradictory reports state that either ARF6, exclusively in the GTP-bound form (using the constitutively active mutant ARF6(Q67L)), pulls down PI(4)P5Kγ from rat brain lysates but not GDP-bound ARF6

(ARF6(T27N) mutant) or GTP-bound ARF1 (ARF1(Q71L) mutant) (Krauss *et al.*, 2003). Conversely, HA-tagged PI(4)P5Kγ pulled down wild type ARF6, ARF6(Q67L) or ARF6(N122I) (another GTP-binding deficient mutant) equally from cotransfected PC12 cells lysates, and even more effectively after provoking an intracellular Ca^{2+} influx with the consequent PKC activation and the apparent dephosphorylation of the lipid kinase (Aikawa and Martin, 2003). This study concluded that interactions between ARF6 and PI(4)P5Kγ appear to be governed principally by the phosphorylation state of PI(4)P5Kγ rather than the guanine nucleotide-bound state of ARF6.

Type II PI(4)P5Ks – PI(5)P4Ks

Substrates. Initially these enzymes were thought to be PI(4)P5Kinases, but later on Rameh *et al.* (1997) showed that the real substrate was not PI(4)P but PI(5)P, and therefore Type II PI(4)P5Ks were truly PI(5)P4Ks. *In vitro*, they are also capable of using PI(3)P as a substrate to generate $PI(3,4)P_2$ and $PI(3,4,5)P_3$ (Hinchliffe *et al.*, 1998a). It is unclear at present whether PI(5)P or PI(3)P is the main physiological substrate of Type II PIPKs, although a PI(3)P4Kinase activity in human platelets, though still unidentified, has been shown not to be a Type II PIPK (Banfic *et al.*, 1998).

Members. As with Type I enzymes, there are three isozymes in this subfamily. The human Type IIα was first cloned in 1995 (Boronenkov and Anderson, 1995; Divecha *et al.*, 1995), whereas the β isoform was identified through a yeast 2-hybrid screen with the juxtamembrane region of the p55 tumour necrosis factor-α receptor (Castellino *et al.*, 1997). The rat γ isoform was cloned by Itoh *et al.* (1998).

Expression. This subfamily of PIPKinases is only found in metazoans. PI(5)P4Kα is found ubiquitously although the highest levels of expression are in brain (Boronenkov and Anderson, 1995). PI(5)P4Kβ can be found in all tissues but its levels are specially higher in skeletal muscle, and again in brain and heart (Castellino *et al.*, 1997). The γ isoform is predominantly expressed in kidney, with low expression in almost all other tissues (Itoh *et al.*, 1998).

Localisation. Both PI(5)P4Kα and β are cytosolic enzymes, and the β isoform can also be found in the nucleus (Heath *et al.*, 2003). PI(5)P4Kγ localises predominantly in the endoplasmic reticulum (Itoh *et al.*, 1998).

Structure. In 1998, Rao *et al.* crystallised and resolved the structure of PI(5)P4Kβ at 3Å, the only member of the PIPKinases whose structure has been determined. As depicted on Figure 1.9, this isoform forms a disc-shaped homodimer, through interactions of its N-terminus, and its structure suggests that an electrostatic mechanism is involved in membrane targeting.

The three isoforms share 60% sequence similarity in their kinase domains. Apart from forming homodimers as seen in the determination of the structure of PI(5)P4Pβ, PI(5)P4Pα has been also shown to form heterodimers with all three isoforms of Type I PI(4)P5K, causing its relocation to the plasma membrane (Hinchliffe *et al.*, 2002).

Function. The function of these PIPKinases is poorly understood. Type II PIPKβ interacts with the p55 TNF receptor in mammalian cells and thus may play a role in TNFα-mediated signalling (Castellino *et al.*, 1997). Since PI(4)P levels in cells far exceed those of PI(5)P (Rameh *et al.*, 1997), the activity of PI(5)P4K would be unlikely to make a major contribution to bulk levels of $PI(4,5)P_2$. Hinchliffe *et al.* (1999b) hypothesised three possible functions for Type II PIPKs. The first is that they act as PI(3)P4Ks to produce $PI(3,4)P_2$. The second is that they are present to

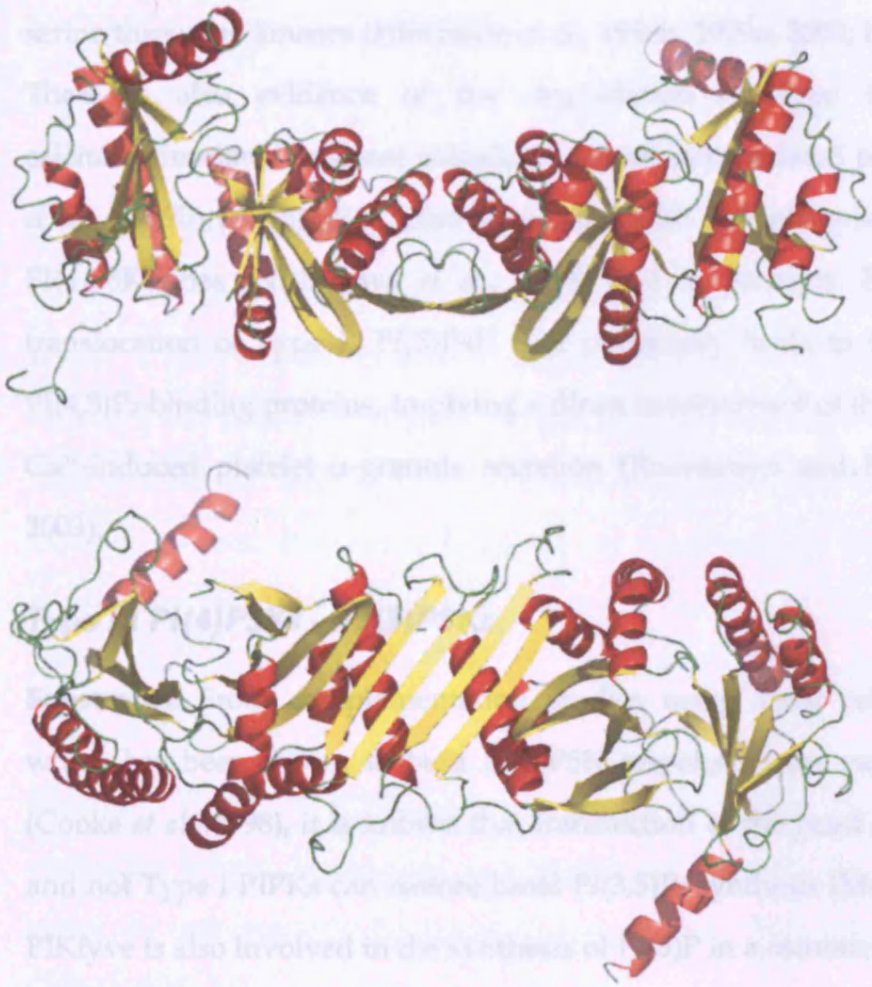


Figure 1.9 Crystallographic structure of a human PI(5)P4Kβ dimer (PDB 1BO1). Sagittal and normal projections relative to the presumed plane of the membrane. The flattened interface of the dimer (inferior surface of bottom projection/front) to interact with membranes (the membrane would be underneath the 3D structure). The bottom view is the same but turned upside down.

clear PI(5)P by transforming it into PI(4,5)P_2. And the third possibility is that they are responsible for the control of a specific pool of PI(4,5)P_2 by being involved in a minor production pathway.

Type II PIPK has been shown to participate in MgATP-dependent, Ca^{2+}-triggered α-granule secretion from platelets, its translocation to the plasma membrane being mediated by PKC (Rozenvayn and Flaumenhaft, 2001, 2003).

Regulation. Unlike Type I PI(4)P5Ks, Type II PIPKs are not stimulated by PA (Loijens *et al.*, 1996a) or regulated by any small G protein (Hinchliffe *et al.*, 1998a). One mode of regulation seems to be by phosphorylation of the lipid kinase by different protein kinases, including casein kinase II and two other

serine/threonine kinases (Hinchliffe *et al.*, 1998b, 1999a, 2000; Huang *et al.*, 2001). There is also evidence of the degradation of Type II PIPKs by the calcium/stimulated protease calpain in thrombin/stimulated platelets (Hinchliffe *et al.*, 1999b). A PI(5)P4K also associates with autophosphorylated PKCμ as PI(4)P5K does (Nishikawa *et al.*, 1998) and in platelets, PKC mediates the translocation of Type II PI(5)P4K that potentially leads to the recruitment of PI(4,5)P_2-binding proteins, implying a direct involvement of the Type II PIPKs in Ca^{2+}-induced platelet α-granule secretion (Rozenvayn and Flaumenhaft, 2001, 2003).

Type III PI(4)P5Ks – PI(3)P5Ks

Substrates. From complementation studies using yeast cells lacking Fab1p, which has been shown to be a PI(3)P5K responsible for vacuole morphology (Cooke *et al.*, 1998), it is known that transfection of the yeast cells with PIKfyve and not Type I PIPKs can restore basal PI(3,5)P_2 synthesis (McEwen *et al.*, 1999). PIKfyve is also involved in the synthesis of PI(5)P in a osmotic response pathway in mammalian cells (Sbrissa *et al.*, 2002).

Members. The sole member of this subfamily of PIPKs was cloned in 1999 by Shisheva *et al.* from a murine adipocyte cDNA library, and it has been termed p235, in reference to its molecular weight, or PIKfyve, due to the presence of an N-terminal zinc-binding FYVE finger.

Expression. Studies at the protein level reveal a widespread distribution among cells and tissues. Differentiated 3T3-L1 adipocytes in culture express the highest protein levels among the cells examined (Shisheva, 2001).

Localisation. The mammalian Type III PIPKs localise to vesicles of the late endocytic pathway, through interaction of their FYVE domain with PI(3)P.

However, other molecular factors placed at post-endosomal compartments must also determine PIKfyve localization, because PIKfyve is undetectable on recycling or early endosomes (Shisheva, 2001).

Structure. Mouse and human PIKfyve harbor several evolutionarily conserved domains with functional significance, including a Zn^{2+}/PI(3)P-binding FYVE finger, a DEP domain of unknown function found in signaling proteins that contain a PH domain, a chaperonin-like region found in proteins implicated in actin and tubulin folding, and a putative catalytic domain found in PI4Ks and PIP5Ks. In fact, PIKfyve belongs to an evolutionary ancient gene family represented by a single-copy gene identified thus far in *S. pombe, S. cerevisiae, C. elegans, A. thaliana, D. melanogaster, M. musculus* and *H. sapiens* (Shisheva *et al.*, 1999), as PI(4)P5Ks do.

Function. These PI(3)P5Ks play a role in membrane trafficking and their enzymatic activity is required to maintain cell morphology by regulating late endocytic membrane homeostasis (Shisheva *et al.*, 1999; Ikonomov *et al.*, 2002; Sbrissa *et al.*, 2002).

Regulation. PIKfyve possesses a protein kinase activity and so it is capable of trans- and autophosphorylation, resulting in inhibition of its 5-kinase activity (Sbrissa *et al.*, 2000).

1.3 ADP-RIBOSYLATION FACTORS

ARF proteins are part of the Ras superfamily of small G proteins. This superfamily is composed of the Rho, Rab, ARF and Ras subfamilies, together with the closely related Gα subfamily. Each one of these subfamilies can in turn be divided into several branches; the ARF subfamily is subdivided into ARF proteins, ARP (ARF-related proteins), ARL (ARF-like proteins) and ARD proteins (ARF-domain proteins), as well as a another group of proteins distantly related to ARF, Sar or Sara proteins. The ARF subfamily comprises at least 30 different members in mammals, 13 in *C. elegans* and 12 in *D. Melanogaster*, indicating that this is a highly conserved family of proteins in eukaryotes with a high degree of expansion (Colicelli, 2004).

ARFs were initially named after their activity as cofactors for cholera toxin-catalysed adenosine diphosphate-ribosylation of the heterotrimeric G protein $G\alpha_s$ (Kahn and Gilman, 1986).

Members

To date, 6 different ARF proteins have been identified in mammals, termed ARF1 through 6, and are classified in three different classes according to their amino acid sequence similarity. Class I comprises ARFs 1, 2 and 3, Class II ARF4 and ARF5 and Class III only ARF6 (Moss and Vaughan, 1995). See Figure 1.10 for a sequence alignment of 2 human isoforms and all the mouse isoforms.

Expression and Localisation

The levels of expression vary depending on the isoform, ARF1 and ARF3 being expressed 3 to 10-fold above those of ARF4-6. All isoforms are expressed in all tissues (Cavenagh *et al.*, 1996). ARF1 has higher levels of expression in kidney,

Figure 1.10 **Alignment of amino acid sequences for mammalian ARFs.**
Identical residues are in red and similar are in blue. The GenBank protein accession numbers of all ADP-ribosylation factors are as follows. *ARF1 human* (CAI23120); *ARF1 mouse* (JC4945); *ARF2 mouse* (JC4946); *ARF3 mouse* (JC4947); *ARF4 mouse* (JC4948); *ARF5 mouse* (JC4949); *ARF6 mouse* (JC4950); *ARF6 human* (AAV38671). The consensus sequences of the Switch I region appears above the alignment in blue and the Switch II region appears in orange. The site of N-terminal myristoylation after co-translational removal of methionine is indicated above the alignment in green. Alignment was generated by **ClustalW** (www.ebi.ac.uk/clustalw) and the printing of the multiple alignment output was performed by **Boxshade** (www.ch.embnet.org/software/BOX_form.html).

liver and placenta; ARF3 in kidney; ARF6 in heart, *substantia nigra*, kidney, and especially high in ovary (Cavenagh *et* al., 1996; Lebeda *et al*, 2003).

Within cells, the distribution of the different isoforms seems to be compartmentalised. ARF proteins, with the exception of ARF6, are predominantly cytosolic and they become bound to membranes upon activation, i.e., GTP-binding. ARF1, ARF2 and ARF3 are mainly cytosolic and are localised at the perinuclear regions corresponding to the Golgi and the ER; ARF1 can also be found in the nucleus. ARF4 and ARF5 are mainly cytosolic, whereas ARF6 is predominantly found bound to the plasma membrane when GTP-bound,

especially in membrane ruffles, or in endosomes when GDP-bound (Peters *et al.*, 1995; Hosaka *et al.*, 1996; Cavenagh *et al.*, 1996; personal unpublished data), although the membrane-bound/cytosolic ratio is variable and dependent on cell type (Yang *et al.*, 1998).

Structure

The amino acid primary structure of ARF proteins of different species has been conserved throughout evolution. Like other small G proteins, they bind GTP to become active and hydrolyse it to become GDP-bound and inactive. Like some other small G proteins, ARF proteins are routinely post-translationally modified. In the case of ARF proteins, this occurs at the N-terminal glycine residue (G2 which becomes G1 once the initiating methionine is removed) with the saturated fatty acid myristate. This modification has been shown to be critical for at least three reasons: firstly, myristoylation increases the nucleotide exchange on ARFs (Franco *et al.*, 1995); secondly, it contributes to the recruitment of ARFs to natural or artificial membranes (Franco *et al.*, 1996); and finally, myristoylation has been demonstrated to be essential for ARF interaction with regulator and effector proteins (Brown *et al.*, 1993; Massenburg *et al.*, 1994).

Single crystal X-ray diffraction analysis of ARF1 (Amor *et al.*, 1994; Greasley *et al.*, 1995; Goldberg, 1998) and ARF6 (Menetrey *et al.*, 2000; Pasqualato *et al.*, 2001) in several forms have revealed important functional regions, depicted in Figure 1.11. Indeed, the overall structure of the ARF molecule consists of seven β-strands, six α-helices and twelve connecting loops arranged in the classical Ras fold. As noted, the feature that distinguishes ARFs from the other Ras family GTP-binding proteins is the N-terminus. This region is an extension of up to 14

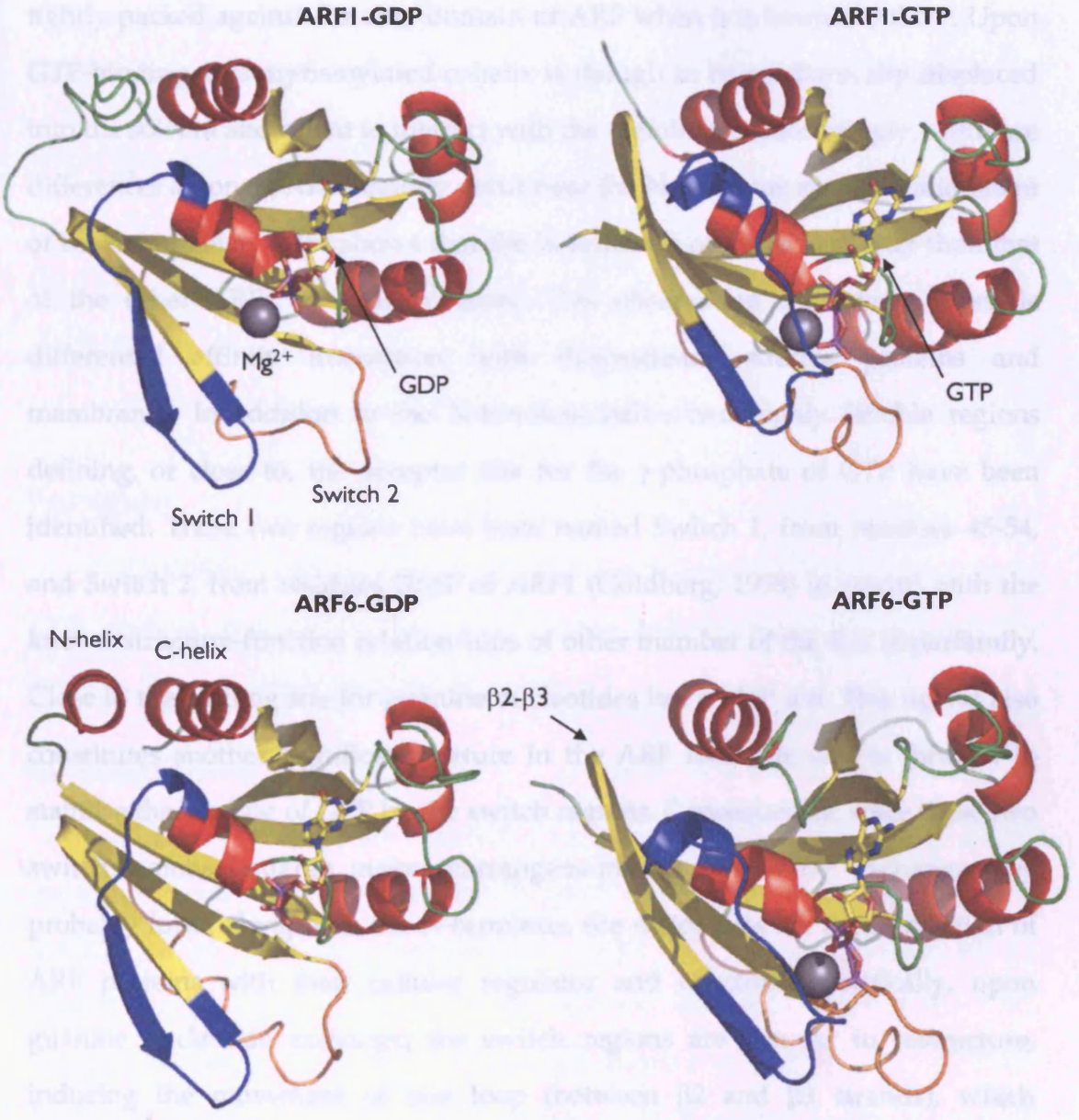


Figure 1.11 **Crystal structures of ARF1-GDP, ARF1-GTP, ARF6-GDP and ARF6-GTP.** Same projection of ARF1 complexed with GDP (bovine GDP-ARF1; PDB code 1RRF), with GTP (mouse GTP-[Δ17]ARF1; PDB code 1O3Y), ARF6 with GDP (human GDP-ARF6; PDB code 1E0S) and with GTP (human GTPγS-ARF6; PDB code 1HFV). The sequence in blue represents the Switch 1 region and in orange represents the Switch 2 region. The grey sphere represents a Mg^{2+} ion.

residues as shown for ARF1 (Greasley *et al.*, 1995) and forms an α-helix that is tightly packed against the core domain of ARF when it is bound to GDP. Upon GTP-binding, this myristoylated α-helix is though to be mechanically displaced into the solvent and orient to interact with the membrane. Interestingly, sequence differences amongst ARFs mainly occur near the N-terminus since the alignment of the mammalian ARFs shows that the N-terminus of ARF6 is shorter than that of the other ARFs by four residues. This observation indicates a possible differential affinity interaction with downstream effector proteins and membranes. In addition to the N-terminal helix, two highly flexible regions defining, or close to, the acceptor site for the γ-phosphate of GTP have been identified. These two regions have been named Switch 1, from residues 45-54, and Switch 2, from residues 70-80 of ARF1 (Goldberg, 1998) in accord with the known structure-function relationships of other member of the Ras superfamily. Close to the binding site for guanine nucleotides lies a Mg^{2+} ion. This ligand also constitutes another significant feature in the ARF molecule and is thought to stabilise the binding of GTP by the switch regions. Consequently, since these two switch regions undergo major rearrangements on GTP↔GDP exchange they probably form, along with the N-terminus, the major sites for the interaction of ARF proteins with their cellular regulator and effectors. Specifically, upon guanine nucleotide exchange, the switch regions are thought to restructure, inducing the movement of one loop (between β2 and β3 strands), which displaces the N-terminal helix from its buried position against the protein core. As a result, the N-terminus exposes its attached myristate tail, which subsequently inserts into the membrane bilayer. Furthermore, the amino acid sequences for switch 1 and switch 2 regions are surprisingly almost identical among the ARF proteins, suggesting that they may have the same conformation

and that they may not be readily distinguishable by the various regulatory and effector proteins that interact with them. It is worth noting that sequence differences between the most distantly related ARF isoforms, ARF1 and ARF6, occur outside the switch regions.

Thus minor differences found in the N-terminus region and the switch regions must presumably account for all of the reported differences in the localisation and function of ARF1 and ARF6. It should be noted that since these two isoforms of ARF are the most extensively studied, especially where side-by-side comparisons are attempted there is a significant bias in the literature.

Function

All ARF proteins are soluble proteins that become membrane bound upon GTP binding. This allows them to colocalise and interact with their effector proteins. Amongst their main functions are:

Membrane Traffic

Class I ARFs appear to control a variety of vesicle transport and fusion steps, which may include budding, transport, and fusion steps in the Golgi, in the ER, and in the endocytotic pathway. ARF1 was originally studied for its function on the Golgi where it localised and also for being responsible for the assembly of coat proteins on membranes (Randazzo *et al.*, 2000). Later, ARF1 has also been implicated in ER-to-Golgi and intra-Golgi transport, transport from the TGN, subsequent endosomal trafficking and the generation and exocytosis of synaptic vesicles and nascent secretory vesicles (Donaldson and Jackson, 2000).

ARF6 on the other hand affects membrane traffic in the cell periphery, in the endosomal-plasma membrane system. It is involved in endosomal recycling to the plasma membrane, receptor mediated endocytosis, regulated secretion,

biogenesis and release of synaptic vesicles (Zheng and Bobich, 2004) and phagocytosis, amongst others (Randazzo *et al.*, 2000; Donaldson and Jackson, 2000).

Actin Cytoskeleton

ARF1 has also been shown to affect the actin cytoskeleton associated with the Golgi apparatus, by recruiting actin and a Golgi-specific isoform of spectrin, independently of COPI recruitment (Godi *et al.*, 1998; Fucini *et al.*, 2000).

ARF6 has been demonstrated to regulate the formation of membrane ruffles or ARF specific protrusions (Radhakrishna *et al.*, 1999; Honda *et al.*, 1999; Boshans *et al.*, 2000); expression of a dominant-negative mutant of ARF6 has been shown to inhibit cell spreading of HeLa cells (Song *et al.*, 1998).

Effector Lipid Metabolising Enzymes

ARF proteins influence the activity of certain lipid metabolising roles in the cellular functions described above. These are PLD and PI(4)P5K. ARF1 and ARF3 were both found to be activators of PLD1 (Brown *et al.*, 1993; Cockcroft *et al.*, 1994), and so were the other ARFs (Massenburg *et al.*, 1994). This activation was potently increased by the presence of the myristoyl group and the N-terminus (Brown *et al.*, 1993, 1995; Massenburg *et al.*, 1994; Zhang *et al.*, 1995). ARF-dependent PLD activity has been detected in plasma membrane, nucleus, Golgi and cytosol (Exton, 1999). The activation of this phospholipase by ARF can be influenced by the participation of several PLD-stimulatory molecules such as PKC, Rho-family GTPases, Ca^{2+}, tyrosine kinases and phosphoinositides (Singer *et al.*, 1995, 1996; Hammond *et al.*, 1997; Exton, 1997, 1999; Houle and Bourgoin, 1999; Zhang Y. *et al.*, 1999).

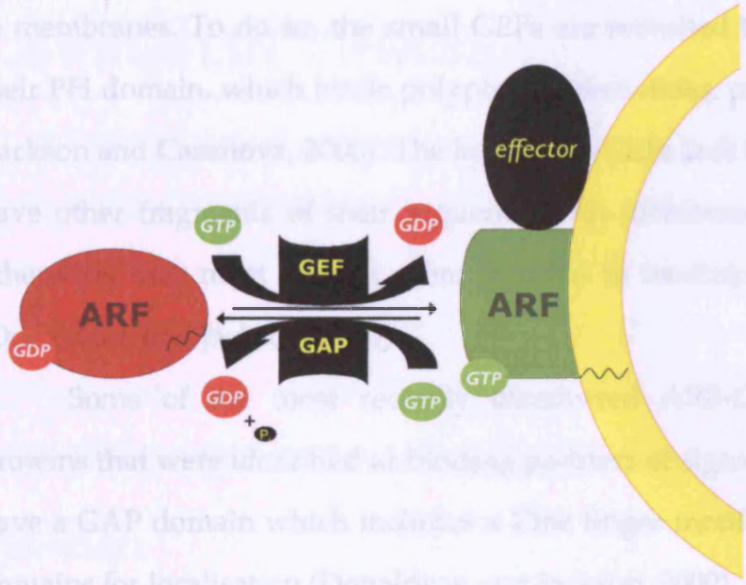


Figure 1.12 Cyclical activation and inactivation of ARF proteins. Replacement of the GDP molecule bound to the inactive cytosolic ARF protein with GTP, aided by an ARF-GEF, releases the hydrophobic N-terminus and the myristate group bound to it, allowing for the molecule to bind to membranes. Inactivation of the GTP-ase and its release from membranes is catalysed by an ARF-GAP that promotes the hydrolysis of the GTP molecule.

The activation of PI(4)P5Kinases by ARF has already been commented on the *Regulation of PI(4)P5K by Small G Proteins* section (pages *55-57*).

ARFs have also been seen to be involved in the recruitment of PI4KIIIβ to Golgi membranes (Godi *et al.*, 1999).

Regulation

ARF proteins are regulated by two types of proteins, ARF guanine nucleotide exchange factors/proteins (GEFs/GEPs), and ARF GTPase activating proteins (GAPs), as shown in Figure 1.12. The first promote a fast and effective activation of ARFs through the displacement of GDP and replacement with GTP; the second activate the hydrolysis of bound GTP to GDP (Donaldson and Jackson, 2000).

ARF-GEFs are divided in two major classes, the large (>100 kDa) ARF-GEFs and the smaller (<100 kDa) ARF-GEFs, on the basis of sequence similarity, functional differences and sensitivity to the fungal metabolite Brefeldin A. ARF activation takes place after both the GEF and its target ARF have been localised

to membranes. To do so, the small GEFs are recruited to target membranes via their PH domain, which binds polyphosphoinositides, particularly PIP_2 and PIP_3 (Jackson and Casanova, 2000). The large ARF-GEFs lack the PH domain, but they have other fragments of their sequence with membrane-targeting information; otherwise, they must rely on other proteins to mediate membrane recruitment (Donaldson and Jackson, 2000).

Some of the most recently discovered ARF-GAPs are multi-domain proteins that were identified as binding partners of signalling molecules. They all have a GAP domain which includes a Zinc finger motif and some also have PH domains for localisation (Donaldson and Jackson, 2000).

1.4 PROTEIN KINASE C

Protein kinase C (PKC) is a multifunctional enzyme that phosphorylates serine and threonine residues in many target proteins. Nishizuka and colleagues were the first to identify it as a protein kinase that phosphorylated histone and protamine in bovine cerebellum (Takai *et al.*, 1977; Inoue *et al.*, 1977). The PKC family is the largest serine/threonine-specific kinase family known to which many cellular responses have been credited (Parker *et al.*, 1992).

Members

The PKC family is represented in all eukaryotes and in *Homo sapiens* comprises several isoforms that have been divided into three subgroups, differing on their cofactor requirements and structure (Dempsey et al., 2000). The **conventional** isoforms, cPKCs, (PKCα, PKCβI and PKCβII and PKCγ) are DAG and phospholipid sensitive and Ca^{2+} responsive. The **novel** isoforms, nPKCs, (PKCδ, PKCε, PKCη and PKCθ) are DAG sensitive but Ca^{2+} insensitive. The **atypical** isoforms, aPKCs, (PKCζ and PKCι/λ) do not respond to either Ca^{2+} or DAG (Parker and Murray-Rust, 2004). An extra two isoforms, PKCμ/PKD (Valverde *et al.*, 1994) and PKCν (Hayashi *et al.*, 1999), both of which are DAG sensitive, have also been identified and could be considered members of a fourth subgroup.

Expression and Localisation

The expression and distribution of PKC isoforms varies markedly between cells and tissues (Nishizuka, 1988). Some isoforms such as PKCα, βI, βII, δ, ε and ζ, are ubiquitously expressed whereas others seem to be restricted to certain tissues; e.g. PKCγ is mainly restricted to the central nervous system and

spinal chord, PKCη is strongly expressed in lung and PKCθ in skeletal muscle (Liu W.S. and Heckman, 1998; Webb *et al.*, 2000).

With the exception of PKCμ, which is a transmembrane protein located at the Golgi (Prestle *et al.*, 1996), PKC enzymes are cytosolic proteins which are recruited to membranes upon stimulation. PKC action can be localised to multiple compartments, including the plasma membrane, (recycling) endosomes, the Golgi and the nucleus. Location is determined in part by the scaffolds but also by targeting information intrinsic to individual isoforms; for example, nuclear localisation/export sequences (NLS/NES) (Parker and Murray-Rust, 2004).

Structure

The general structure of a PKC molecule consists of a catalytic and a regulatory region found at the C- and N-termini respectively, as illustrated in Figure 1.13. Both regions are composed of a number of conserved domains interspersed with regions of lower homology. These domains are a kinase domain, a C2 or C2-like (in the case of novel PKCs) domain, a C1 domain, a pseudosubstrate domain and a PB1 (Phox and Bem 1) domain in the case of atypical PKCs. The kinase domains are closely related and form part of the AGC kinase superfamily. PKCμ and PKCν family members (not included in the structure figure) have distinct kinase domains but some related regulatory properties. cPKCs respond to Ca^{2+} through an archetypal C2 domain; nPKCs contain a C2-related domain that does not retain Ca^{2+}-coordinating residues. The C1 domain of cPKCs and nPKCs contain two cysteine-rich regions and serve them to bind DAG and phorbol esters. In contrast, aPKCs have altered C1 domains and are not DAG sensitive; regulation occurs in part through the N-

terminal PB1 domain (Liu W.S. and Heckman, 1998; Parker and Murray-Rust, 2004).

Structures for C1, C2 and PB1 domains have been determined (Figure 1.13), and kinase domain models based on the highly related PKA have been built (not shown).

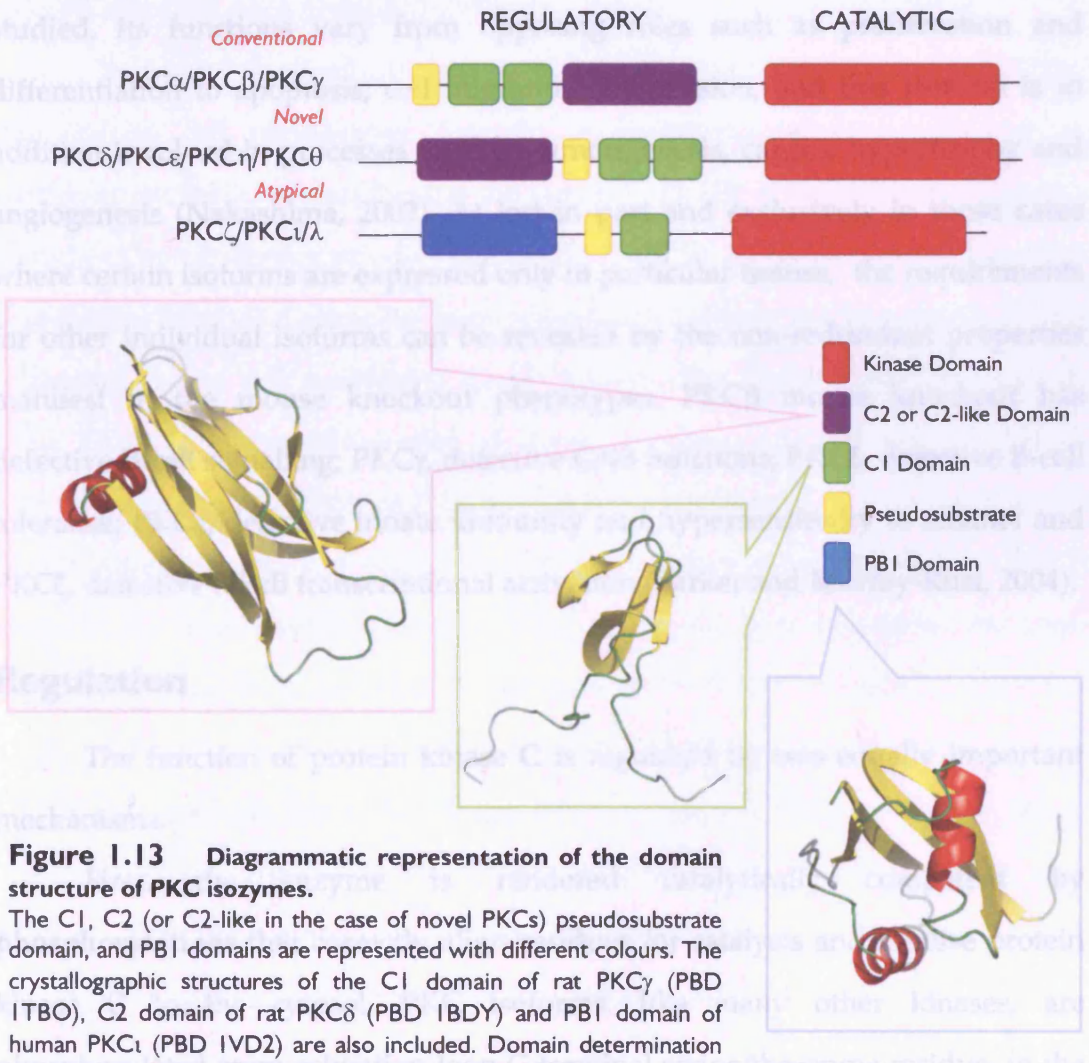


Figure 1.13 **Diagrammatic representation of the domain structure of PKC isozymes.**
The C1, C2 (or C2-like in the case of novel PKCs) pseudosubstrate domain, and PB1 domains are represented with different colours. The crystallographic structures of the C1 domain of rat PKCγ (PBD 1TBO), C2 domain of rat PKCδ (PBD 1BDY) and PB1 domain of human PKCι (PBD 1VD2) are also included. Domain determination was generated using **CDD** (Marchler-Bauer *et al.*, 2003).

Function

Since its discovery, much interest has been shown in PKC and its role in signal transduction. Development, differentiation, proliferation and carcinogenesis are all crucial processes for which PKC has been implicated (Webb *et al.*, 2000), as well as others such as receptor desensitization, modulation of membrane structure events, regulation of transcription, mediation of immune responses and learning and memory among many others (Newton, 1995). In the case of PKCα, the most widely expressed of all isoforms and possibly the most studied, its functions vary from opposing roles such as proliferation and differentiation to apoptosis, cell migration to adhesion, and this isoform is in addition involved in processes such as tumorigenesis, cardiac hypertrophy and angiogenesis (Nakashima, 2002). At lest in part and exclusively in those cases where certain isoforms are expressed only in particular tissues, the requirements for other individual isoforms can be revealed by the non-redundant properties manifest in the mouse knockout phenotypes. PKCβ mouse knockout has defective B-cell signalling; PKCγ, defective CNS functions; PKCδ, defective B-cell tolerance; PKCε, defective innate immunity and hypersensitivity to ethanol and PKCζ, defective NFκB transcriptional activation (Parker and Murray-Rust, 2004).

Regulation

The function of protein kinase C is regulated by two equally important mechanisms.

First, the enzyme is rendered catalytically competent by **phosphorylations** that correctly align residues for catalysis and localise protein kinase C to the cytosol. PKC isoforms, like many other kinases, are phosphorylated at an activation-loop C-terminal serine/threonine residue, in the

sequence TFCGTP by PDK1. This is followed by an additional phosphorylation, probably PDK-mediated, or autophosphorylation at a more C-terminal serine/threonine residue in the sequence FSY/FTY. Nineteen amino acids N-terminal to this phosphorylation motif is another autophosphorylation site, the so called TP site (Bornancin and Parker, 1996; 1997; Stempka *et al.*, 1997; Li *et al.*, 1997). The PDK1-dependent, activation-loop phosphorylation occurs in conjunction with C-terminal phosphorylations to lock the kinase domains in their active conformations; there remains some debate as to the order and requirements for these phosphorylations.

The second mechanism involves **translocation** from the cytosol to binding domains at cell membranes. Specific anchoring proteins immobilized at particular intracellular sites localise the kinase to its site of action. These proteins include receptors for activated C-kinase (RACKS), adducins, annexins and other cytoskeletal proteins. Following an increase in intracellular calcium levels, cPKCs interact with the cell membrane in an inactive, but conformationally distinct form. DAG facilitates penetration of these isozymes into the cell membrane. When attached, the affinity of PKC for calcium is increased such that activation of the enzyme is achieved, depending on its phosphorylation status. For nPKCs isoforms, no equivalent mechanism promoting interaction with DAG has been elucidated. For cPKCs, the initial recruitment event is the Ca^{2+}-sensitive step. For aPKC isoforms activation can be driven in part by interaction with the Cdc42-GTP–Par6 complex, which binds the PB1 domain (Parker and Murray-Rust, 2004). Phosphatidylserine is the membrane lipid anchor for both cPKCs and nPKCs, although other membrane phospholipids may ultimately link extracellular signals to intracellular events through PKC (Newton, 1995).

Chapter Two

Aims and Objectives

The main objective of this thesis was to investigate some of the aspects of the control of enzymes involved in polyphosphoinositide metabolism.

Initially, I aimed to determine whether some specificity of interaction between different isoforms of ARF GTPases and phosphatidylinositol 4-phosphate 5-kinases exists. With this in mind, a biochemical approach was undertaken in an attempt to show differences in activation by these small G proteins in simplified cell-free systems using recombinant proteins. As a compliment permeabilised cells were also used to try and bring the biochemistry closer to cellular systems. Finally, I wanted to demonstrate a physical interaction between the two proteins using coprecipitation assays.

Another potential regulator of PI(4)P5Ks is PKC, according to a sequence analysis that predicts a hypothetical phosphorylation site within the lipid kinase. With this in mind, I tested the effect of the presence of PKC on the activity of PI(4)P5K. As part of this investigation the effect of different lipids on the activity of PI(4)P5Ks were tested leading to the unexpected discovery of the activation of the lipid kinase by diacylglycerol. A major aim was the characterisation of this new effect and comparison to the activation by the best described lipid-activator phosphatidic acid.

Finally, I wanted to determine whether ARF proteins also had an effect in the related group of lipid kinases PI3Ks (Class I). p110γ appeared to be strongly influenced by the GTPase and therefore I aimed to characterise this effect using a similar approach to that used with PI(4)P5K.

Chapter Three

Materials and Methods

3.1 MATERIALS

Reagents and Compounds

- [γ-^{32}P]ATP, CDNB and potassium phosphate buffer (part of the GST Detection Module), CNBr-activated Sepharose 4 FastFlow beads, Glutathione Sepharose 4B beads and Phenyl Superose (HR10/10) column were purchased from Amersham Biosciences.
- Agarose, bovine serum albumin, ammonium sulphate, benzamidine, bromophenol blue, culture media RPMI-1640 and DMEM, diC8:0-PA and diC8:0-DAG, EGTA, goat serum, HEPES, kanamycin, DL-α-dimyristoyl-phosphatidylcholine, myristic acid, nalidixic acid (sodium salt), PMA, PMSF, R59949 inhibitor, sheep anti-goat HRP-conjugate secondary antibody, SDS, sodium phosphate, streptolysin-O, TEMED, Tris-base, Tris-HCl, Trypsin-EGTA and Triton X-100 were from Sigma.
- Heat-inactivated foetal calf serum was from Imperial Laboratories.
- Ampicillin, antipain, aprotinin, ATP (disodium salt), GTPγS, leupeptin, Nutridoma-SP and pepstatin were from Roche.
- Acetic acid, acetone, calcium chloride solution, chloroform, EDTA (disodium salt), glycerol, glycine, hydrochloric acid, imidazole, IPTG, lysozyme, magnesium chloride solution, methanol, PBS, potassium oxalate, reduced glutathione, sodium azide, sodium chloride and sodium hydroxide were from BDH Laboratory Supplies.
- 40% Acrylamide/Bis solution, APS, ethidium bromide, goat anti-mouse and goat anti-rabbit HRP-conjugate secondary antibodies and Supported Nitrocellulose membrane (0.2 μM) were from Bio-Rad.
- Coomassie Brilliant Blue R250 was from Fluka.

- DTT and mouse anti-ARF 1D9 monoclonal antibody from Alexis Biochemicals.
- Ni-NTA (Fast-Flow) and Polyfect Transfection Reagent from Qiagen.
- LB broth, penicillin (5000 Units/ml), streptomycin (5000 μg/ml) and PKCα (old Panvera) were from Invitrogen.
- VectaSpin Micro 10 μm Polypropylene Mesh filters, filter paper grade 1 and Silica Gel TLC 60 plates from Whatman (Fisher).
- Tissue culture flasks and dishes from Helena Biosciences.
- All synthetic diacylglycerols, phosphatidic acids, phosphatidylinositol, phosphatidylinositol 4-phosphate and phosphatidylinositol-4,5-bisphosphate were from Avanti Polar Lipids.
- Recombinant DGKζ and anti-DGKζ antibody were obtained from Dr Matt Topham (Huntsman Cancer Institute, University of Utah School of Medicine, Salt Lake City, Utah, USA).

Cell Lines

- Cos7 cells, monkey kidney cells transformed with SV40 virus T antigen, were from American Type Culture Collection, USA (www.atcc.org).
- HEK293 cells, human embryo kidney cells transformed with sheared human Ad5 DNA, were from the European Collection of Animal Cell Culture, UK (www.ecacc.org.uk).
- HL60 cells, human Caucasian promyelocytic leukaemia cells, were purchased from the European Collection of Animal Cell Culture, UK.
- Clone 12CA5 hybridoma cells were a kind gift from Dr. Leo Price (Netherlands Cancer Institute (NIWI), Amsterdam, Netherlands)

3.2 METHODS

Mammalian Cell Culture

Cos7 and HEK293 Cells

Cos7 cells and HEK293 cells were cultured in DMEM medium supplemented with 10% heat-inactivated foetal calf serum, penicillin (50 units/ml) and streptomycin (50 μg/ml). Cells were grown typically in 100 mm ∅ culture dishes until confluent or 90% confluent respectively, in a humidified atmosphere containing 5% CO_2 at 37°C. Cells were passaged as required by their state of confluence by removal of the medium, gentle wash with PBS followed by addition of 2 ml of Trypsin-EGTA. After 5-10 minutes, 20 ml of medium was added to the cells before they were dispersed with a pipette. Cells were then split into new dishes at a desirable dilution (generally 1/10) and 20 ml of fresh medium was added.

HL60 Cells

HL60 cells were cultured typically in 50 ml of RPMI-1640 medium supplemented with 10% heat-inactivated foetal calf serum, penicillin (50 units/ml) and streptomycin (50 μg/ml). Cells were grown to a density of approximately $1x10^6$ cells/ml in a humidified atmosphere containing 5% CO_2 at 37°C. Cells were passaged every 48 hours (after collection by centrifugation) at a split ratio of 1:5 again in 50 ml of medium.

Plasmid Construction

Murine PI(4)P5K Bacterial Expression Plasmid pGEX-4T-2-PI(4)P5Kα, β and γ (GST-fusion proteins) and Mammalian Expression Plasmid pEBB-PI(4)P5Kα-HA

pGEX-4T-2-PI(4)P5Kα, β and γ and pEBB-PI(4)P5Kα-HA were the kind presents of Dr Chris Carpenter (Harvard Medical School, Boston, Massachusetts, USA).

Construction of the Murine PI(4)P5Kα Mammalian Expression Plasmid pEBG-PI(4)P5Kα (GST-fusion protein)

DNA coding for PI(4)P5Kα was subcloned from the pGEX-4T-2-PI(4)P5Kα plasmid into the mammalian expression vector pEBG. BL21(DE3)pLysS bacteria transformed with the parent plasmid were grown overnight at 37°C in 10 ml of LB broth supplemented with ampicillin (100 μM). A miniprep of the culture (Qiagen's QIAprep Spin Miniprep Kit, according to the manufacturer's instructions) provided sample DNA. Both the pGEX-4T-2 and the pEBG plasmids were digested sequentially with Bst BI (65°C for 2 hours) and Not I (37°C for 2 hours) with DNA being recovered after the first step with GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences) as indicated by the manufacturer. The products of the consecutive digestions were analysed in a 1% agarose gel in order to confirm the presence of the right insert and the linearised pEBG. The band corresponding to the insert (~2 kb) was cut out of the gel and the DNA extracted from the agarose polymer using GeneClean III kit (Q-BioGene, according to the manufacturer's instructions).

The insert was ligated into the new vector in a reaction catalysed by T4 DNA ligase (0.5 μl of 400 U/ml ligase) diluted in ligation buffer (250 mM Tris (pH

7.6), 50 mM $MgCl_2$, 5 mM ATP, 5 mM DTT, 25% (w/v) PEG-8000) for 30 minutes at room temperature. Competent *E. coli* DH5α cells were then transformed with the new plasmid and plated on ampicillin-containing agarose. 5 colonies were picked from the plate and grown in 5 ml of LB broth and ampicillin overnight. Minipreps were made from each colony and the DNA was double-digested using the restriction enzymes Sal I and Not I for 1 hour at 37°C. The products of the digestion were run on a 1% agarose gel and two bands of the anticipated size confirmed the proper transformation of the bacteria with the expected construct.

Construction of the ARF Bacterial Expression Plasmid pMon5840-ARF1-$(His)_6$ and pMon5840-ARF6-$(His)_6$

DNA coding for ARF proteins was amplified by PCR from authentic samples using forward and reverse primers that coded for the termini of the sequence and any desired extensions. These extensions comprised 5′ sequence for a Nco-1 restriction site and 3′ sequence containing codons for six consecutive histidine residues followed by a Hind III restriction site. For example, the following primers were used for bovine ARF1:

N-terminus (sense strand)

5′ – TATATACC·ATG·GGG·AAT·ATC·TTT·GCA·AAC·CTC – 3′

C-terminus (antisense strand)

5′ – ATTATTAAGCTTCA·GTG·ATG·ATG·ATG·ATG·ATG·TTT·CTG·
GTT·CCG·GAG·CTG·ATT·GGA·CAG – 3′

Restriction sites are in boxes, ARF1 sequence and reverse compliment ARF1 sequence are shown in blue, the stop codon is in red and the 6xHis coding sequence is in green. Related primers, but with sequence corresponding to fragments of ARF1 or full-length ARF6, were designed along similar lines with all restriction sites and other features as for the ARF1 primers. PCR products

were digested with Nco I and Hind III, purified and ligated into a sample of plasmid pMon5840 (kind gift of Dr JI Gordon, Washington University Medical School, Missouri, USA) which had been previously cut with the same two restriction enzymes. After transformation into *E. coli* XL1 Blue cells and selection by ampicillin resistance, plasmid DNA was isolated from positive clones and the insert sequenced to exclude any mutations. Plasmids were then transformed into *E. coli* BL21-DE3 pLysS cells with or without co-transformation with expression plasmid pBB131 coding for the yeast *N*-myristoyltransferase 1 (NMT1, kind gift of Dr JI Gordon, Washington University Medical School, Missouri, USA) and selected by ampicillin or ampicillin *plus* kanamycin resistance respectively. Positive or double positive clones were expanded and stored as frozen glycerol stocks until required.

pXS-ARF1(wt)/ARF1(T31N)/ARF1(Q71L) and pXS-ARF6(wt)/ ARF6(T27N)/ARF6(Q67L) (all HA-tagged) Plasmids

pXS plasmids coding for HA-tagged ARF1(wt), ARF1(T31N), ARF1(Q71L), ARF6(wt), ARF6(T27N) and ARF6(Q67L) were the kind gift of Dr Julie Donaldson (NIH, Bethesda, Maryland, USA).

Expression and Purification of Recombinant Proteins

Expression and Purification of GST fusion PI(4)P5Kα, β and γ in E. coli

Recombinant Type I phosphatidylinositol 4-phosphate 5-kinase α, β and γ were prepared by methods similar to those described previously (Tolias *et al.*, 1998a).

For the expression and purification of PI(4)P5K proteins *E. coli* BL21(DE3)pLysS bacteria were transformed with pGEX-4T-2-PI(4)P5Kα, β or γ plasmids. Use of these plasmids, where the insert is fused with the catalytic domain of the enzyme glutathione-S-transferase (GST), allows for easy recovery using beads coated with glutathione. Since the pGEX-4T-2 plasmid carries ampicillin resistance all cultures of the singly transformed bacteria can be selected at all points by use of this antibiotic at a suitable dilution (100 μg/ml).

An initial culture of transformed cells was grown in 5 ml of LB broth overnight at 37°C in presence of 100 μM ampicillin. Cells from the overnight culture were expanded in two conical flasks containing 200 ml of LB, in the presence of ampicillin, and allowed to grow overnight at 37°C. The next day the cells were expanded in four 1-litre flasks of LB without ampicillin for 3 hours. Expression of PI(4)P5K was then induced with 0.5 mM IPTG and cells were left to express for an extra 3 hours. They were then harvested by centrifugation at 4420x*g* for 10 minutes at 4°C using a JA-10 fixed angle rotor in a J2-21 Centrifuge (Beckman). The cell pellet from a 4-litre culture was then resuspended in a total volume of 80 ml of lysis buffer 1 (50 mM Tris (pH 8.0), 40 mM EDTA, 25% (w/v) sucrose, 0.02% (w/v) NaN_3, 1 mg/ml lysozyme) and incubated with gentle stirring for 30 minutes at room temperature. Cells were lysed by the addition of 32 ml of lysis buffer 2 (50 mM Tris (pH 8.0), 100 mM $MgCl_2$, 0.2% (w/v) Triton X-

100, 0.02% (w/v) NaN_3). In addition, 1 mM DTT, 1 mM PMSF, 10 μg/ml antipain, 2 μg/ml pepstatin, 1 μg/ml leupeptin and 1 μg/ml aprotinin were added to the suspension, which was then incubated for 30 minutes at 4°C with stirring. The broken cells were subsequently homogenised by using a Potter-Elvehjem homogeniser. The homogenate was clarified by ultracentrifugation at 10,000x*g* for 60 minutes at 4°C using a Type 35 Beckman rotor in a Sorvall Ultracentrifuge (OTD 65B model). In the meantime, 4 ml of 50% slurry of Glutathione Sepharose 4B beads was washed three times in 25 ml of lysis buffer 1 and the final 2 ml of beads was resuspended in 3 ml of lysis buffer 1+2 (10:4 ratio). Supernatant containing PI(4)P5K protein was then split into three 50 ml-tubes and the GST-beads divided equally, and the mixture left rotating overnight at 4°C.

The beads were washed into *dialysis buffer* (50 mM Tris, 50 mM NaCl and 5 mM $MgCl_2$, pH 7.5, 1 mM DTT and 1 mM PMSF, pH 7.6 adjusted at 4°C) 3 times by centrifugation and resuspension before being collected together. Protein was eluted with three 5 ml washes of 10 mM reduced glutathione in dialysis buffer by rotating for 15 minutes at 4°C. The three elution products were pooled and dialysed to remove the glutathione (see *Protein Dialysis* and following sections for more details on protein analyses).

Expression and Purification of His-tagged ARF1 and ARF6 in E. coli

Recombinant ARF1 and ARF6, both myristoylated and nonmyristoylated, as well as [Δ17]ARF1 were prepared as previously described (Randazzo *et al.*, 1995) with a few modifications. In the case of [Δ17]ARF1 $(His)_6$, its preparation did not require the co-translational addition of myristic acid as it is an ARF1 mutant that lacks the first 17 amino acids of its N-terminus and therefore the myristoylation site.

E. coli BL21(DE3)pLysS bacteria were transformed with either pMon5840-ARF1-$(His)_6$ or pMon5840-ARF6-$(His)_6$, along with pBB131 (yeast NMT1). NMT catalyses the *in vivo* transfer of a myristate group to a glycine residue at position 2 of the ARF protein. Bacteria lack this type of transferase activity, hence its coexpression with the different plasmids coding for ARF proteins under those circumstances where myristoylation is required, allows for the addition of the fatty-acyl chain to the N-terminus of the protein. A 6X His-tag was inserted into the sequence for ARF1, ARF6 and [Δ17]ARF1 at the C-terminus as described above. Since the pMon5840 plasmids carry ampicillin resistance and pBB131 carries kanamycin resistance all cultures of the double or singly transformed bacteria can be selected at all appropriate points by combinations of these antibiotics, e.g. ampicillin (100 μg/ml) and kanamycin (50 μg/ml).

An initial culture of transformed cells was grown in 5 ml of LB overnight at 37°C in presence of ampicillin and kanamycin as required. Cells from the overnight culture were expanded in two conical flasks containing 200 ml of LB, once again containing antibiotics, and allowed to grow overnight at 37°C. The next day the cells were expanded in four 1-litre flasks of LB for 3 hours in the absence of antibiotics. 12.5 ml of a freshly prepared solution containing myristic acid (2.52 mg/ml) and BSA (20 mg/ml) was added to each flask. At the same, time, the expression of NMT, where required, was induced by addition of 1 mM IPTG and left to express for an additional hour. When required the temperature was then reduced to 26°C to optimise the yield of myristoylated proteins as previously shown by Franco *et al.* (1995). Nalidixic acid (25 mg/ml) was then added to induce ARF protein expression (1.25 ml of the freshly prepared nalidixic acid solution was added to each flask). After 3 hours of protein expression, further myristic acid and BSA was added and the culture was then allowed to grow overnight at 26°C. The cells were harvested by centrifugation at

4420x*g* for 10 minutes at 4°C and the cell pellet from the 4-litre culture was resuspended in a total volume of 80 ml of lysis buffer 1 (50 mM Tris (pH 8.0), 25% (w/v) sucrose, 0.02% (w/v) NaN_3, 1 mg/ml lysozyme) and incubated with gentle stirring for 30 minutes at room temperature. Cells were then lysed by addition of 32 ml of lysis buffer 2 (50 mM Tris (pH 8.0), 100 mM $MgCl_2$, 0.2% (w/v) Triton X-100, 0.02% (w/v) NaN_3). In addition, 1 mM DTT and PMSF were added to the suspension which was then incubated for 30 minutes at 4°C with stirring. The cells were subsequently homogenised and the homogenate was clarified by ultracentrifugation at 10,000x*g* for 60 minutes at 4°C. ARF proteins were then purified from the supernatant through two steps. Firstly, the supernatant obtained after ultracentrifugation was pumped through a 25 ml-bed volume column of Ni-NTA fast-flow beads with a Minipuls II pump (Gibson) at about 2 ml/min. The column was previously equilibrated with the following cycle: 100 ml of Nickel column buffer containing 20 mM NaH_2PO_4, 20 mM Tris, 300 mM NaCl, 0.2% (w/v) NaN3, pH 6.0, followed by 100 ml of the same buffer supplemented with 0.5 M imidazole and then followed by 50 ml of a mixture (5:2 ratio) of lysis buffers 1 and 2 respectively. The Ni-NTA column was then connected to an FPLC system which was set at a flow rate of 2.5 ml/min. After a washing step, the protein was eluted with a continuous linear gradient of 0 to 0.5 M imidazole in the Nickel column buffer. This particular column chromatography step was performed at room temperature or at 4°C with identical results. Fractions of 5 ml were collected. In a second chromatographic step, myristoylated ARFs were resolved from the underivitised forms by hydrophobic interaction chromatography on phenyl superose, if required, as follows. The pooled samples from the Ni-NTA column were mixed with solid ammonium sulphate to a final concentration of 1 M. In the cold, the pH was adjusted to 7.6 and the sample clarified by centrifugation and passage through a

0.45 μM filter. The HR10/10 Phenyl Superose column was equilibrated in a cold Tris buffer (20 mM Tris (pH 7.6) and 0.02% (w/v) NaN_3) containing 1 M $(NH_4)_2SO_4$. After passage of the sample through the column, the bed was washed with one volume of buffer containing 1.7 M $(NH_4)_2SO_4$. The column was then developed with a decreasing linear ammonium sulphate gradient in the same Tris buffer from 1 M $(NH_4)_2SO_4$ and 8 ml-fractions were collected. All flow rates were 0.5 ml/min or less. Proteins elution was monitored by absorbance at 280 nm. Protein-containing fractions were dialysed against several changes of Tris buffer (100:1 volume ratios as above) in the cold and then concentrated as described for other samples and stored frozen in small aliquots.

Alternatively, the myristoylated protein could be collected by differential ammonium sulphate precipitation. Peak fractions from the Ni-NTA chromatography step containing the ARF protein mixture were pooled and dialysed against 1.7 M $(NH_4)_2SO_4$ (pH 7.6) overnight at 4°C. The pellet, containing the myristoylated proteins were dissolved in salt-free buffer and then dialysed to remove the highly concentrated salt (see *Protein Dialysis* and following sections for more details on protein analyses).

We could not detect biochemical difference between forms of myristoylated ARFs prepared by either of these two methods.

The extent of myristoylation of the protein preparations was assessed by reverse phase HPLC as myristoylated forms of the proteins show a characteristic increase in retention time.

Nucleotide-preloaded recombinant ARF proteins were obtained from Dr Neil Justin (MRC NIMR, Mill Hill, London).

Expression and Purification of GST fusion and HA-tagged Proteins in Mammalian Cells

Several proteins were expressed and purified from mammalian cells after their transient transfection. For this matter, Cos7 cells were used most commonly, although HEK293 cells could be used as well with exactly comparable results. For the expression in Cos7 cells, the plasmids pEBB-PI(4)P5Kα-HA (product used in most PI(4)P5K activity assays), pEBG-PI(4)P5Kα (GST-fusion protein used in protein-interaction assays) and pXS-ARF1(wt), pXS-ARF1(T31N), pXS-ARF1(Q71L) pXS-ARF6(wt), pXS-ARF6(T27N) and pXS-ARF6(Q67L) (all C-terminal HA-tagged) were employed.

Transfections were performed using Qiagen's Polyfect Transfection Reagent, exactly as indicated by the manufacturer's instructions. In some cases and exclusively with HEK293 cells, calcium phosphate precipitation could also be utilised as an alternative method to produce recombinant proteins. In both cases, after 48 hours of expression cells were washed in PBS and lysed using the so called low salt lysis buffer (50mM Tris (pH 8), 1% (v/v) NP-40 and 1 mM PMSF) on ice, after assessing different buffers and concluding that this buffer did not interfere with the activity of PI(4)P5K. Cells were then scraped from the plate, sonicated to ensure total lysis and the insoluble debris removed by centrifugation at 4°C for 30 minutes on a bench top Eppendorf centrifuge at 14,000x*g*.

Addition of 100 µl of 50% slurry of either anti-HA antibody-coated sepharose beads or Glutathione Sepharose beads to each lysate (per 100 mm cell culture dish equivalent) allowed the efficient immunoprecipitation (HA-tagged) or affinity purification (GST fusion) of the recombinant proteins after 1 hour of rotation at 4°C. It was then straightforward to wash the beads 3 times into lysis

buffer and a final time into kinase assay buffer (in the case of PI(4)P5K), for immediate assessment of PI(4)P5K activity or storage of small aliquotes at -20°C. In the case of protein-interaction experiments, the whole lysate was frozen as such or in aliquots for its later use in coprecipitation assays.

Alternatively, both the GST fusion and the HA-tagged kinases could be eluted from the beads prior to activity assays, by performing a 1-hour elution at 4°C using either 10 mM reduced glutathione or 2 mg/ml of HA peptide (NH_2-YPYDVDYA-COOH) together with 2 mg/ml of BSA in kinase activity assay buffer. Dialysis was not required since neither the glutathione (at concentrations equal or lower than 10 mM) nor the HA peptide interfered significantly with the assay once further diluted into the reaction mixture.

General Protein Handling

Protein Dialysis to Remove Glutathione, Imidazole or Ammonium Sulphate

Protein samples to be dialysed, for instance after elution of a protein from beads during its purification or its precipitation with ammonium sulphate, were transferred into a soaked dialysis tubing of 12 kDa molecular weight cut-off (Sigma) which was then carefully clamped and placed into a 5 litre-container filled with dialysis buffer (above) in the case of PI(4)P5K or 20 mM Tris (pH 7.6) in the case of ARF, at a volume about 100 times the volume of the protein sample. The container was then placed in a cold room set at 4°C. To facilitate equilibration of high salts, a spin bar was placed in the bottom of the container. The protein sample was dialysed for 24 hours with gentle stirring. Changes of buffer were performed 2 more times giving a one million-fold theoretical dilution.

Protein Concentration

In order to reduce the total volume in which a protein is dissolved, the sample was introduced into a properly preassembled 50 ml capacity Amicon concentrator (Millipore) containing a 10,000-MW cut-off Amicon ultrafiltration membrane (YM10 Millipore). The ultrafiltration membrane was placed in the bottom of the concentrator with its glossy side up. A nitrogen gas head at an operating pressure read between 20 psi (1.4 kg/cm^2) and 30 psi (2.1 kg/cm^2) was applied to the concentrator during stirring.

Consequently, further concentration of the protein could also be achieved using 10 kDa molecular weight cut-off Microsep microconcentrators (Pall, Gelman Laboratory) in order to get a smaller volume of protein sample (<0.5 ml). A maximum of 3.5 ml of protein sample was transferred into the sample reservoir of the microconcentrator which was then centrifuged at 3000x*g* using a JA-20 fixed angle rotor (Beckman) for 30 minutes at 4°C.

Protein Quantification

The concentrated protein samples of bacterial origin were quantified by using a dye-binding assay based on the method of Bradford (Bio-Rad or Pierce kits). The amount of protein in the sample was measured according to the manufacturer's instructions. The protein samples were read using an Ultrospec II spectrophotometer (LKB Biochrom) with reference to a BSA standard curve.

Protein Storage

Recombinant proteins expressed in bacteria were dated and stored in 30% glycerol at -20°C for short term storage and -80°C for long-term storage.

Protein Electrophoresis

SDS-PAGE was used to resolve different proteins, such as PI(4)P5K and ARF, according to their molecular weight. A 1-mm thick, 10% separating gel for PI(4)P5K and 12.5% for ARF was made, both with a ≤5% discontinuous stacking gel. The separating gel was made first, by mixing 10 ml of separating buffer (1.5 M Tris (pH 8.8), 0.4% (w/v) SDS) for both 10 and 12.5% gels, with 20 or 17.5 ml of water respectively, 10 or 12.5 ml of 40% acrylamide/bis solution, 400 µl of 20% (w/v) APS and 30 µl TEMED. The separating gel was then poured between the slabs of the preassembled SE 600 vertical slab gel unit (Hoefer) in the dual gel casting stand to a level about 4 cm from the top of the gel. A few microlitres of water-saturated isobutanol was added on top of the separating gel to eliminate any air bubbles and prevent oxygen diffusion. While polymerisation of the separating gel was taking place, the stacking or upper gel was prepared by mixing 5 ml of stacking buffer (0.5 M Tris (pH 6.8), 0.4% (w/v) SDS) with 12.7 ml of water, 2 ml of 40% acrylamide/bis solution, 100 ml of 20% (w/v) APS and 10 ml TEMED. After polymerisation of the separating gel, the small excess of alcohol was poured off and the surface of the polymerised separating gel washed 3 times with distilled water. The stacking gel was then poured in on top of the separating gel. A 1 mm thick, 20-well comb was subsequently inserted, taking care not to trap any air bubbles below the teeth of the comb. The gel was allowed to sit for at least 30 minutes. After polymerisation, the comb was gently removed from the stacking gel and the newly formed wells were filled with tank buffer (0.025 M Tris, 0.192 M glycine, 0.1% SDS (pH 8.3)). The whole slab gel unit, which includes the gel, was introduced in the electrophoresis tank, half full with tank buffer, after assembling and filling an upper buffer chamber.

Protein samples were mixed with 1/6 of their volume of 6X SDS sample buffer and then heated to about 80°C for 5 minutes and then loaded in the wells.

The SE 600 vertical slab gel unit was then connected to an Electrophoresis Power supply (Model 3000 Xi, Bio-Rad) and the gel run for 14 hours (overnight) at 7.5 mA constant current per gel.

Western Blotting

Western blotting was performed for identification and quantification of recombinant PI(4)P5K and ARF proteins using the following protocol. The SDS-PAGE gel containing the resolved protein bands following electrophoresis was cut off and washed 2 times in transfer buffer (25 mM Tris, 192 mM glycine, 10% (v/v) methanol for PI(4)P5K and 20% for ARF) for 15 minutes each time. A similarly equilibrated semi-dry transfer system was applied for the protein gel in a Multiphor II Electrophoresis System (Pharmacia Biotech). For each gel, one sheet of 0.2 μm pore nitrocellulose filter membrane (Bio-Rad) and 12 sheets filter paper (Whatman) were cut the same size as the gel and soaked in transfer buffer. Prior to protein transfer, a transfer sandwich was assembled on the anode plate in the following order: 6 sheets of filter paper, nitrocellulose membrane, SDS-PAGE gel and 6 sheets of filter paper. The Multiphor II Electrophoresis System was connected to a power supply (2303 Multidrive XL, LKB Bromma). The protein sample was transferred to the nitrocellulose membrane for 1 hours using 0.8 mA/cm^2 current density. After protein transfer, the nitrocellulose membrane was incubated in blocking solution containing 5% (w/v) skimmed milk powder in 100 ml TBS-Tween buffer (100 mM Tris, 0.9% (w/v) NaCl, 0.1% (w/v) Tween-20) for 1 hour at room temperature on a lab shaker (gently rocking). After blocking, the membrane was washed 3 times in 100 ml TBS-Tween for 5 minutes each time. PI(4)P5K and ARF proteins were then detected by incubating the

membrane with a monoclonal primary antibody (see Table 2.1 for types of antibodies and working dilutions) diluted in TBS-Tween buffer for 1 hour at room temperature. After 3 buffer washes in TBS-Tween, the membrane was subsequently incubated with secondary antibody HRP (horseradish peroxidase) conjugate diluted in 100 ml TBS-Tween containing 5% (w/v) skimmed milk powder and 1% (v/v) goat serum (when applicable) for 1 hour at room temperature. The membrane was then washed 3 times in TBS-Tween for 5 minutes each time and subsequently incubated with 10 ml of an HRP substrate solution made up of one volume of Stable Peroxide Solution and one volume of Luminol/Enhancer Solution (West Pico Chemiluminescent substrate, Pierce) for 10 minutes at room temperature. Following the incubation time with chemiluminescent reagents, the membrane was laid flat between sheets of clear plastic and developed by short exposure in the FujiFilm Intelligent Dark Box. Images were captured, stored electronically and the amount of protein quantified using Aida software.

Antibodies						
PRIMARY			SECONDARY			
ANTI-	ORIGIN	DILUTION	ANTI-	ORIGIN	DILUTION	SERUM
PI(4)P5Kα	rabbit	1/1,000	Rabbit	goat	1/3,000	1% goat serum
GST	goat	1/3,000	Goat	sheep	1/10,000	NO serum
HA	mouse	1/200	Mouse	goat	1/3,000	1% goat serum
ARF (1D9)	mouse	1/2,000				

Table 2.1 Antibodies and their dilutions used in different Western blots.
The table shows the primary monoclonal antibodies, their origin and the working dilution (in TBS-Tween buffer), as well as the corresponding secondary antibodies, along with their origin and working dilution. Indication of the use of 1% goat serum is also included in those cases where it is applicable.

Transformation and Transfection

Preparation of Competent Bacteria for Transformation

For bacterial cells to take up a plasmid, they need to be made competent. For this matter, a 2 ml-overnight culture of *E. coli* XL1 Blue or DH5α in LB broth was expanded in 200 ml of the same medium, and left to grow for approximately 2 hours, until the optical density value (A_{600}) was of around 0.32. Then, cells were centrifuged at 6,000x*g* for 10 minutes at 4°C, resuspended in 10 ml of ice-cold 0.1 M $CaCl_2$ and left standing on ice for 10 minutes. The previous step was repeated once more and afterwards cells were centrifuged again and resuspended in 4 ml of $CaCl_2$. Cells were then competent and ready for transformation with the desired plasmid.

Heat Shock Transformation of Competent Bacteria

For transformation of bacteria with a desired plasmid, 100 µl of competent cells were mixed with the plasmid in question (approximate amount 0.1 µg) and left standing on ice for 10 minutes. The cells were then heat-shocked at 42°C for 90 seconds and returned to ice for an extra 10 minutes. After that, 5 ml of LB broth was added to the cells and these were incubated at 37°C for 90 minutes. The majority of the supernatant was removed after centrifugation and the cells resuspended in the remaining 200 µl and plated onto a LB-Agar plate with ampicillin (100 µM) and left to grow overnight at 37°C. The following day 5 colonies were selected from each plate and grown in 5 ml of LB with ampicillin overnight at 37°C. The following day minipreps were made to check that the bacteria had been transformed with the correct construct, using Marligen Bioscience's *High Purity Miniprep System* (as indicated by the manufacturer's instructions). This assessment could be done by digesting the plasmid with

suitable restriction endonucleases and displaying the plasmid on an an agarose gel or more accurately by sequencing.

General DNA Handling

DNA Electrophoresis

Linearised fragments of DNA could be run in an agarose gel to confirm their size and identity by comparison with a DNA ladder, composed of various fragments of DNA of known length or number of bases. The 1% agarose gels were made by mixing 100 ml of TAE buffer (121 g of Tris-base, 28.55 ml of glacial acetic acid, 18.6 g of Na_2-EDTA·$2H_2O$ and 500 ml of distilled water, adjusted pH 8.5) with 1 g of agarose and heated in a standard microwave until all the agarose was dissolved. 2 µl of ethidium bromide (10 mg/ml) was then added to the solution and this was poured into a casting tray to solidify, together with a well comb. Once solid, the gel could be inserted into a submarine electrophoresis apparatus and submerged in TAE buffer. The samples to analyse were mixed with 10X loading buffer and added onto the wells, and run at 100 V for approximately 20 minutes. Once the samples reached the end of the gel, this could be extracted and DNA visualised under UV light to localise and identify the separated fragments.

Experimental Assays

PI(4)P5K Activity Assay

This is an *in vitro* assay that follows the production of $PI(4,5)P_2$ from PI(4)P and [γ-^{32}P]ATP. For this assay, GST- or HA-tagged PI(4)P5K (any of the three isoforms) expressed in *E. coli*, Cos7 or HEK293 cells could be used, bound to or eluted from beads. Enzymes prepared in 10 µl reaction buffer (50 mM Tris pH

7.5, 50 mM NaCl, 5 mM $MgCl_2$ and 1 mM DTT), diluted to a predetermined concentration that would give a detectable signal, were mixed with 10 µl sonicated substrate vesicles (typically final 340 µg/ml DMPC, 40 µg/ml PI(4)P, 1 mM sodium deoxycholate, 1 mM HEPES and 10 µM EDTA), on ice. Reaction buffer or 10 µl of ARF proteins and/or 10 µl of the non-hydrolysable GTP-analogue GTPγS (both diluted in the same buffer), as required, was added to give a total volume of 40 µl. Each reaction was started, after preincubation in a water bath if required, with the addition of 10 µl of ice-cold MgATP (50 µM final), ~0.185 MBq [γ-^{32}P]ATP, $MgCl_2$ (5 mM final) and DTT (1 mM final) and transferred to a water bath at the required temperature. Reactions were terminated after 20 minutes in most cases with 80 µl of 1M HCl plus 180 µl of methanol:chloroform (1:1) and thorough mixing. The emulsion was resolved into two phases by centrifugation and 10-40 µl of the organic phase spotted on a silica gel 60 TLC plate pretreated with potassium oxalate and preheated. Spots of [^{32}P]$PI(4,5)P_2$ were clearly resolved using chloroform:acetone:methanol:acetic acid:water (46:17:15:14:8) as the mobile phase and identified through comigration of standards. Radioactivity was quantified on a Fuji Bas phosphorimager.

PI3K Activity Assay

This *in vitro* assay is equivalent to the PI(4)P5K activity assay but using 10 µl of PI3K Iα, Iβ, Iδ or Iγ (both p101/p110γ or 130 nM on its own). DMPC:PI(4)P vesicles are substituted by sonicated PI vesicles generated in the kinase activity assay buffer. $PI(4,5)P_2$ vesicles could also be used to asses kinase activity. Recombinant p110γ was obtained from Prof Peter Shepherd (Department of Biochemistry, UCL, London), recombinant p101/p110γ from Dr Len Stephens (Babraham Institute, Cambridge) and recombinant p110α/β/δ from Prof Mike Waterfield (Ludwig Institute for Cancer Research, UCL, London).

PI(4)P5K Assay in SLO-Permeabilised HL60 Cells

In order to be able to gain access to the interior of the cell, where the interaction and activation between PI(4)P5K and ARF proteins takes place, it was necessary to permeabilise HL60 cells using the toxin streptolysin-O (SLO). SLO is a cytolytic protein secreted by the genus of bacteria *Streptococcus*, which specifically binds to cholesterol present in the plasma membrane and penetrates it, by generating transmembrane channels or pores of between 25 and 30 nm in diameter (Bhakdi *et al.*, 1985). At the concentrations used in this study SLO generally preserves the architectural integrity of the cells, but allows the loss of soluble components of the cytosol through these pores, and not those cellular components that have strong interactions with membranes or the cytoskeleton. Thus way, the reintroduction into cells of specific low molecular weight agents, such as ARF or GTPγS, becomes possible and allows for the study of their influence on the activity of PI(4)P5Ks.

After 48 hours of growth, 40 ml from a 50 ml HL60 cell culture was centrifuged and resuspended in 978 µl of kinase activity assay buffer together with 20 µl of SLO (final 0.4 IU/ml) and 2 µl of EGTA (final 0.2 mM). EGTA was included in the buffer to chelate any free calcium released during permeabilisation. The cells were permeabilised for 10 minutes at 37°C. Proteins such as ARF were shown to leak out of the cells within 5-10 minutes (Fensome *et al.*, 1996). At the end of this step, the permeabilised cells were sedimented by centrifugation at 3000x*g* for 5 minutes to remove the cytosolic components that had leaked out during permeabilisation. The permeable HL60 cells were then resuspended in kinase buffer once more. For time course experiments the cells were then split into 4x400 µl tubes, together with 200 µl of buffer and 10 µl of 1M DTT, as well as 200 µl of ARF (1 µM final), GTPγS (10 µM final) and/or buffer on

ice. The mixes were left standing on ice for 5 minutes to allow for the different components to diffuse inside the cells. The phosphorylation reaction was started by transfer of the tubes into a 37°C water bath along with synchronised addition of 200 µl of [γ-^{32}P]MgATP (same final concentration as in the standard kinase assay). Triplicate samples were taken out of each tube at established time-points and transferred into tubes containing 80 µl of 1M HCl in order to halt the reactions. The rest of the procedure coincides exactly with the standard PI(4)P5K activity assay. The amount of PIP, PIP_2, PIP_3 and PA yielded during the incubation was established by differential migration on the TLC plate. The identity of spots on the TLC plates was confirmed by HPLC analysis or co-migration of standard samples.

A variation of this assay is used in Chapter 5 (below) at fixed-time points (10 minute) with recombinant PI 3-kinase (p110γ) in half of the samples, as well as nonmyristoylated isoforms of ARF1 and 6 and a N-terminal truncated isoform of ARF1. All other constituents were used at the same final concentration but divided from the beginning into separate 50 µl-final reaction volumes (per triplicate). The reactions were conducted as if performing a PI(4)P5K activity assay. All tubes carried 10 µl of cell resuspension and 10 µl of MgATP, together with 10 µl of ARF proteins, 10 µl of GTPγS and/or 10 µl of diluted p110γ (130 nM stock) substituted with buffer under those conditions where one or several of these components were absent from the mixture.

Protein Interaction Experiments

In an attempt to demonstrate the existence of a physical association of PI(4)P5K and ARF proteins, the following method was developed. 100 µl of HA antibody-coated packed beads was initially blocked with 1 ml of lysis buffer containing 5 mg/ml of BSA for 1 hour at 4°C. The supernatant was removed and

the beads resuspended in fresh BSA-containing lysis buffer, total volume of 1 ml. In order to immunoprecipitate the HA_6-tagged ARF proteins, 50 µl of the pre-blocked 50% bead slurry was added to 300 µl of cells lysates containing recombinant ARF (typically one millilitre of lysate corresponded to the whole lysed content of a transfected 100 mm-dish), alongside a control tube composed of 50 µl of slurry solution and 300 µl of 1 mg/ml HA peptide (to block the antibody) and 0.1 mg/ml BSA in lysis buffer, and left to rotate at 4°C for 1 hour. Subsequently, the beads were washed 3 times in lysis buffer (plus BSA) and 25 µl of GST-fusion PI(4)P5Kα-containing lysate. Another 175 µl of lysis buffer (plus BSA) was added to the beads. Recombinant HA-ARF loaded beads and PI(4)P5Kα lysates were left to rotate at 4°C for 1 hour, after which the beads were washed 3 more times in lysis buffer and a final time in kinase activity assay buffer. After resuspension of the beads in a final volume of 100 µl of kinase activity assay buffer, 10 µl were used to asses the associated lipid kinase activity (standard PI(4)P5K activity assay) and the remaining 90 µl were used to quantify the amount of ARF (using anti-HA antibody), PI(4)P5Kα (using anti-PI(4)P5Kα antibody) and final amount of beads after extensive handling (by measuring the final amount of HA antibody present per sample using the anti-mouse HRP-conjugate secondary antibody). These values were used to normalise the ARF-associated PI(4)P5Kα protein mass and activity.

In Vitro Effect of PKCα on PI(4)P5Kα Activity

To evaluate the effect of the protein kinase PKCα on the activity of soluble PI(4)P5Kα expressed in bacteria, the following protocol was developed. First, it was necessary to combine and preincubate both enzymes with the required PKCα-activating effectors (see Chapter 4). In a total volume of 30 µl, 15 µl of soluble PI(4)P5Kα and 1 µl of PKCα (1/10 dilution of 0.11 mg/ml stock) had to be

preincubated together for 60 minutes at 37°C in the presence of 6 µl of 375 µM ATP and 6 µl of PS/DAG/Ca2+ solution (500 µg/ml PS, 100 µg/ml DAG, 1 mM HEPES (pH 7.4), 0.03% (w/v) Triton X-100, 0.02% NaN_3 and 500 µM $CaCl_2$) and 2 µl of standard PI(4)P5K activity assay buffer to make up the volume.

After allowing time for potential phosphorylation to occur, the lipid kinase could be extracted from the mixture by affinity purification using glutathione sepharose beads. 50 µl of packed beads was added along with kinase activity assay buffer and rotated for 1 hour at 4°C. Consequently, all PKC effectors could be removed from contact with PI(4)P5Kα to avoid their direct interference in its activity. To achieve this the beads were first introduced into 10 µm-pore microfilters (Whatman) to minimise losses of small quantities of beads through manipulation. The activity of the associated lipid kinase could then be assessed either on the beads or after its elution through the microfilters using 10 mM reduced glutathione.

Finally, normalisation of the results became imperative as handling of the beads provided differences in the recovery of the different samples in any one experiment. Since the lipid kinase was generated as a GST-fusion protein, its associated glutathione S-transferase activity could be used as a direct indicator of protein concentration. Such activity was proportional to the quantifiable rate of formation of a yellow product after addition to the protein solution of 1 mM final concentration of reduced glutathione, 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) and 10 mM potassium phosphate buffer (pH 6.5) at room temperature (according to the Amersham *GST Detection Module* protocol). The rate of appearance of the yellow product was measured in a plate reader at 340 nm, and the kinase activity values normalised against these rates.

Alternatively, the same assay could be performed by substitution of the soluble PI(4)P5Kα from bacterial origin for enzyme expressed in mammalian

cells. In this case, HA-tagged PI(4)P5Kα was used associated with beads. The preincubation and quantification of the kinase activity was performed as before, without eluting the lipid kinase from the beads due to the limited yield of this process.

In Vivo Effect of PA/DAG/PMA on PI(4)P5Kα Activity

Cos7 cells were grown to the appropriate confluence for transfection (according to Qiagen's Polyfect Transfection Reagent instructions) in fifteen 35-mm dishes, and twelve of them were transfected with the pEBG-PI(4)P5Kα or pEBB-PI(4)P5Kα-HA plasmids. After 48 hours of expression, the cells were gently washed with PBS, and 500 µl of fresh DMEM medium were added (to the 3 untransfected control and 3 transfected control dishes), along with 1 mM diC8-PA (3 transfected dishes), 1 mM diC8-DAG (3 transfected dishes) or 100 nM phorbol myristate acetate (PMA) (3 transfected dishes). The cells were left to incubate at 37°C for 30 minutes, after which the medium was removed, the cells washed with PBS and lysed using 500 µl of the previously described standard low salt lysis buffer, on ice. The lysates were collected in eppendorf tubes, sonicated to ensure complete cell lysis and cellular debris removed by centrifugation. All steps were performed at 4°C. The recombinant kinase was then extracted out of the supernatant as described previously, with 30 µl of 50% slurry of either glutathione sepharose beads or HA-antibody coated beads, as appropriate, washed and resuspended in 100 µl final volume of kinase activity assay buffer. 10 µl samples were used to assay kinase activity and the remaining 90 µl to quantify the amount of kinase recovered through western blotting in order to normalise the activity against the amount of kinase expressed/recovered.

Neutrophil Migration Assay

Isolation of Neutrophils. Human whole blood was collected by ante-cubital venipuncture into acid citrate dextran, ACD (4.2%, (w/v) disodium hydrogen citrate-1,5-hydrate and 5%, (w/v) D-glucose). 20 ml of blood was mixed with an equal volume of 6-hydroxyethyl starch (eloHAES) and 5 ml 10% (v/v) ACD (ACD diluted 1:10 (v/v) in 0.9 % (v/v) NaCl) and incubated for 45 min to allow sedimentation. The upper layer containing the leukocyte rich buffy coat was removed and centrifuged at 600x*g* for 10 min at 18°C. The resulting supernatant was discarded except for 2 ml in which the cell pellet was resuspended. Percoll diluted 10:1 (v/v) with 9% (v/v) NaCl, was designated 100% percoll. This was then further diluted with PBS to produce 80%, 70% and 55% (v/v) percoll solutions. Gradients were prepared with 4 ml 80% percoll as the base layer, 4 ml 70% percoll as the middle layer and 3 ml 55% percoll as the top layer. The resuspended cell pellet was gently layered on top of the percoll gradient and centrifuged at 1100x*g* for 25 minutes at 18°C. This procedure separated the neutrophils held at the 80/70% interface and they were then washed three times in HBSS. The cells were counted using a haemocytometer and Kimura counter-stain, and resuspended at $2x10^6$ cells/ml in neutrophil buffer (HBSS supplemented with 20mM HEPES (pH 7.2) and 0.1% BSA). The neutrophils were left to recuperate for 1 h prior to chemotaxis.

Measurement of Chemotaxis. Chemotaxis of neutrophils was measured in a 48 well modified Boyden chamber (Neuro Probe Inc.) by modification of standard methods (Wilkinson, 1998; Matsushima *et al.*, 1989). Neutrophils were resuspended at a concentration of $2x10^6$ cells/ml in neutrophil buffer. Chemoattractant peptide fMLP (10nM) in neutrophil buffer was loaded into the lower wells, so that the wells were filled with a slight convex meniscus (approximately 29µl), taking care to ensure that no air bubbles were present in the wells. For each compound dissolved with the chemoattractant, 6 wells were filled. A 3-µm pore cellulose nitrate filter, pre-soaked in neutrophil buffer was placed over the lower wells. The rubber gasket was placed onto the filter and the upper block was clamped over the lower block. The chamber was then covered

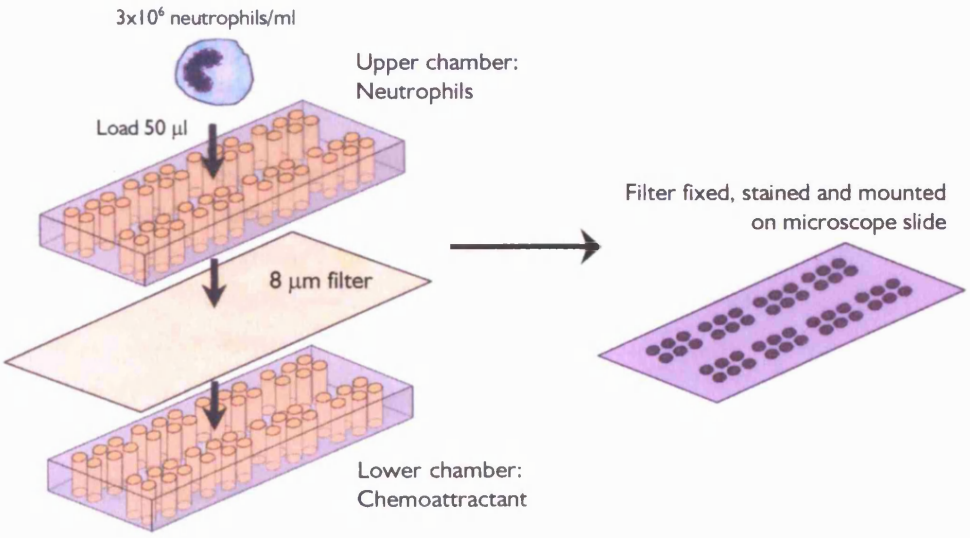


Figure 2.1 **An overview of measurement of chemotaxis**
Chemoattractant (29µl) is loaded into the bottom wells and a cellulose nitrate filter is placed on top. A rubber gasket is placed over the top of the filter and the upper chamber is clamped on top. The chamber is preincubated for 15 min at 37°C and 50µl neutrophils at 3 x 10^6 are loaded into the top well. The chamber is then incubated for 90 min at 37°C before the filter is removed. The filter is then fixed and stained and mounted on microscope slides prior to cell counting

with a glass slide to prevent evaporation and pre-incubated at 37°C for 15 min. Following preincubation, 50 µl neutrophil cell suspension was loaded into each of the upper wells; again care was taken to ensure there were no air bubbles present in the wells. Together with the cells, Brefeldin A (5 µg/ml), wortmannin (100 nM), butan-1-ol or butan-2-ol (1% v/v) were also loaded to assess their effect on migration. Controls with DMSO (<0.2 % v/v) were also tested in both the upper and lower chambers to discount any effect caused by the solvent. The chamber was incubated at 37°C for 60 min to allow migration of the cells through the filter. The filter was then removed and the cells in the filter fixed in 70% (v/v) ethanol. The neutrophil nuclei were stained with haematoxylin and the filter dehydrated with a series of alcohol steps as follows:

Alcohol Dehydration		
Solution	Time	Effect
70% (v/v) ethanol	5 min	Fixative
Distilled H_2O	1 min	Wash
Haematoxylin	45 sec	Stain
Distilled H_2O	1 min	Wash
Tap water	2-3 min	Wash
70% (v/v) ethanol	2 min	Dehydration
95% (v/v) ethanol	2 min	-
8:2 ethanol:butanol	5 min	-

Table 2.2 Protocol for dehydration of the cellulose nitrate filter.
List of washes with different compounds and the corresponding incubation times for dehydration of the cellulose nitrate filter for visualisation and quantification of embedded cells.

The cellulose nitrate filters are opaque, and must be cleared in xylene before mounting onto glass slides. Once the filter had been dehydrated it was soaked in 100% (v/v) xylene overnight. The filter was cut into 4 sections and mounted onto glass slides using DePex mounting medium (EMS). A glass coverslip was applied, making a waterproof seal to prevent the filter from rehydrating. The same field was chosen at the centre of each well and cells were counted *blind* to limit observer bias (40x objective, 6x eyepiece). All cells that had migrated more than 20 μm into the filter were counted. This is termed the leading front method. An overview of measurement of chemotaxis is presented in Figure 2.2. Studies by Wilkinson (1998) have shown that the leading front method yields consistently closer replicates for each concentration of chemoattractant used. Figure 2.2 indicates two possible migration patterns of cells through the filter. At a set point in the filter 20μm there may be an equal number of cells in the presence of two different concentrations of chemoattractant (A and B). However, the number of cells in the filter after this point will vary. This means that two different concentrations of chemoattractant

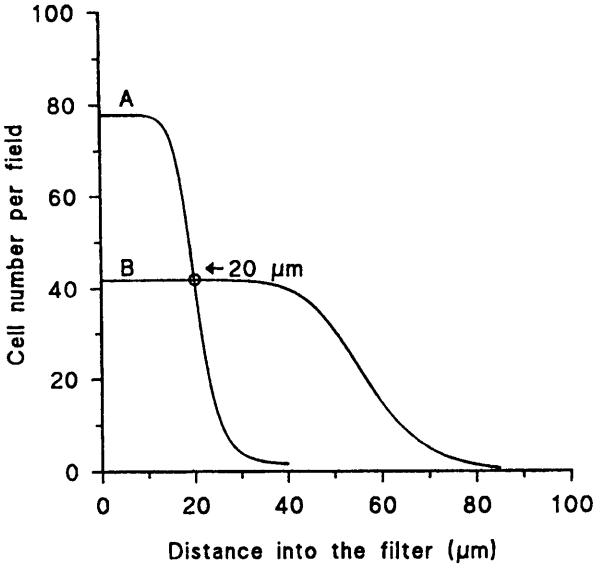


Figure 2.2 Example of different migration patterns of neutrophils towards a chemoattractant
A and B represent the migration patterns of neutrophils through a 120 μm filter in the presence of two different concentrations of chemoattractant. The arrow indicates the same number of cells at the given distance of 20 μm.

may give the same apparent cell migration at a chosen distance, while in reality the total number of cells migrating through the filter is different. The leading front method allows all the cells in the filter beyond a set point to be counted, in order to indicate the actual migration of the cells into the filter. The start point is chosen where there are enough cells per field to represent the population (approximately 1-200 cells), but where each cell is distinct from neighbouring cells. In all chemotaxis experiments, the start point was standardised at 20 μm from the top of the filter.

Preparation of Compounds

Preparation of Lipid Mixtures for Kinase Activity Assays

The standard lipid mixture for PI(4)P5K activity assay was prepared by mixing in an eppendorf tube 1.7 mg of DMPC and 100 μl of 2 mg/ml PI(4)P. Subsequently, the chloroform was evaporated under nitrogen gas and 500 μl of 10 mM sodium deoxycholate and 500 μl of 10 mM HEPES (pH 7.5) and 0.1 mM EDTA solution were used to resuspend the lipids by thorough sonication and vortexing (yielding a final concentration following dilution in the standard assay mixture of 40 μM PI(4)P and 500 μM DMPC). The 1 ml of lipid stock solution was kept at -20°C.

Variations to this lipid mixture were also used to assess the influence of different lipids in the activity of PI(4)P5K. In such cases, the preparation procedure was the same, nevertheless the amount of PI(4)P was generally increased to 80 μM final to equal that of the other lipids under study. The effect of the presence or absence of DMPC and deoxycholate or Triton X-100 was also determined by using different combinations of lipids with DMPC and detergents.

the external medium and thus simplify the purification process by reducing the amount of soluble proteins in the supernatant. Cells were grown until they senesced and started dying. The supernatant was then collected after sedimentation of the cells and the soluble antibody concentrated by ammonium sulphate precipitation (75% saturation) at 4°C during a minimum period of 4 hours. The precipitate was collected by centrifugation and then dialysed over 24 hours with three changes of 5 litres of 0.1M $NaHCO_3$ and 0.5M NaCl at 4°C. The final protein concentration was adjusted to 5 mg/ml before coupling to sepharose. CNBr-activated Sepharose 4 FastFlow beads were used, after their activation by 10 consecutive washes with 50 ml of ice cold 1mM HCl (according to the manufacturer's instructions). The beads and the antibody were mixed together and left to rotate overnight at 4°C, leading to efficient coupling. The remaining reactive groups were then blocked with 0.05M glycine (pH 8), and the beads aliquoted and stored at -20°C until needed.

Preparation of 6X SDS Sample Buffer

6X SDS sample buffer (0.376 M Tris, pH 6.8, 12% SDS, 60% (w/v) glycerol, 0.6 M DTT, 0.06% (w/v) bromophenol blue) was prepared by diluting 9.4 ml of 1 M Tris (pH 6.8) into 15.6 ml of water in a beaker. Then, 15 g of liquid glycerol, 3 g of SDS, 15 mg of bromophenol blue and 2.3 g of DTT was added to the Tris buffer and allowed to dissolve. Another extra 15 g of glycerol was finally added after complete dissolution of the components, and aliquots of 6X SDS sample buffer were finally stored at -20°C.

Expression of Data

All results presented are from individual experiments repeated at least three times with similar results, unless otherwise stated. Within each of these

individual experiments all discrete measurements were made as triplicates and occasionally quadruplicates, with the exception of the protein-interaction experiments where single replicates were used. Data and statistical analysis were performed by SigmaPlot 8.0 and Excel software. Statistical significance of the values was evaluated by Student's *t*-test. Oftentimes it was necessary to describe the significance of statistical relationships between various different measurements within one experiment. Under these circumstances, precise *p* values are described in the accompanying text.

The analyses of the amino acid sequences of the proteins of interest described in this thesis were conducted as described in the legends of the corresponding figures and tables. Further analysis was performed to determine the extent of sequence identity between representative members of a particular protein, using ClustalW (www.ebi.ac.uk/clustalw) and Boxshade (www.ch.embnet.org/software/BOX_form.html). Domain information was obtained using the Conserved Domain Database or CDD (www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

Molecular models of proteins obtained by crystallography were rendered using PyMOL 0.97 Software (DeLano, W.L. The PyMOL Molecular Graphics System (2002) (www.pymol.org). Coordinates were retrieved from PDB.

Chapter Four

Regulation of Type I PI(4)P5K by ARF Proteins

4.1 INTRODUCTION

There are six known mammalian ARF family members which have been classified in three different groups according to their size and sequence similarity. Class I is composed of ARF1, ARF2 and ARF3, ARF4 and ARF5 form Class II and ARF6 is the sole member of Class III (Moss and Vaughan, 1995). This group of small GTP-binding proteins are post-translationally modified with the addition of a myristate group on a glycine (G2) on their N-terminus by *N*-myristoyltransferase (NMT) (Khan *et al.*, 1988). This acyl chain is indispensable for the effective binding of ARF to membranes where it needs to be localised to fulfil its function.

ARF1, ARF5 and ARF6 have been shown to be activators of Type I PI(4)P5K, but there have been no exhaustive comparative studies aiming to clarify whether there is any preferred combinations of G-protein or kinase isoforms. Using a bacterial co-expression system based on the yeast myristoyl-CoA:protein NMT coexpression system of Duronio *et al.* (1990), recombinant ARF1 and ARF6 were synthesised in *E. coli* in their fully myristoylated or nonmyristoylated forms. These purified proteins were used to investigate the comparative activation of Type I PI(4)P5Ks using both cell-free and permeabilised-cell assays. Finally, wild type ARF proteins along with constitutively active and dominant-negative mutants were expressed in mammalian cells and used for precipitation experiments to try and prove a direct interaction between ARF and Type I PI(4)P5K and explore the properties of the interaction.

4.2 RESULTS

Purification Of ARF Proteins And PI(4)P5Ks

ARF proteins purified from bactera were subjected to two consecutive purification steps. ARF proteins obtained from cell lysis were first loaded on Ni-NTA column and eluted with a linear gradient of imidazole (Figure 4.1). A single symmetric peak for each ARF isoform was obtained and the corresponding fractions under this pead were pooled and loaded on a penyl Supersoe column in

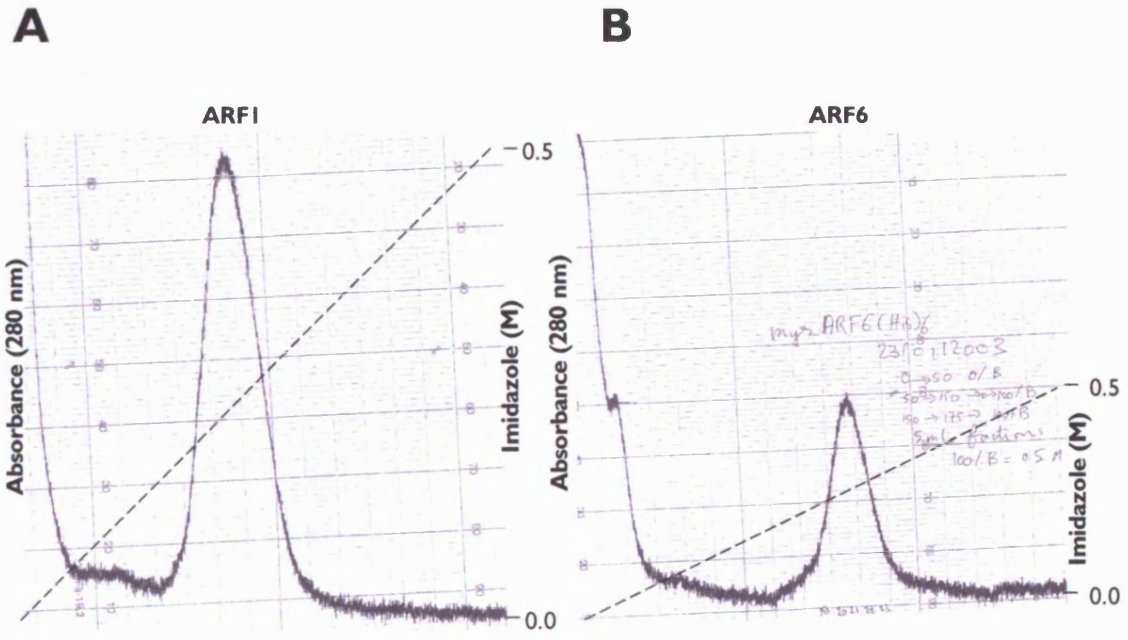


Figure 4.1 Elution of ARF proteins from Ni-NTA column.
The supernatant obtained after cell lysis was applied to a 25 ml bed volume column of Ni-NTA, which had previously been equilibrated in 100 ml of Nickel column buffer (20 mM NaH_2PO_4, 20 mM Tris, 300 mM NaCl, 0.2% NaN_3 (w/v), pH 6.0) followed by 100 ml of the same buffer supplemented with 0.5 M imidazole and then followed by 50 ml of a mixture (5:2 ratio) of lysis buffers 1 and 2 respectively (see Materials and Methods section for more details). Proteins were eluted with a linear gradient of 0 to 0.5 M imidazole in the Nickel column buffer. Fractions of 5 ml were collected. Protein elution was monitored by absorbance at 280 nm.

a second chromatographic step in which myristoylated ARFs were resolved from the underivitised forms by hydrophobic intection chromatography. Example of chromatogram of phenyl Superose fractionation step in ARF1 purification is shown in Figure 4.2. The peaks of myristoylated ARF1 and nonmyristoylated ARF1 are indicated.

The extent of myristoylation of the protein preparations was determined by reverse phase HPLC. The myristoylated forms of the proteins showed a characteristic increase in retention time. The profile of myristoylated and

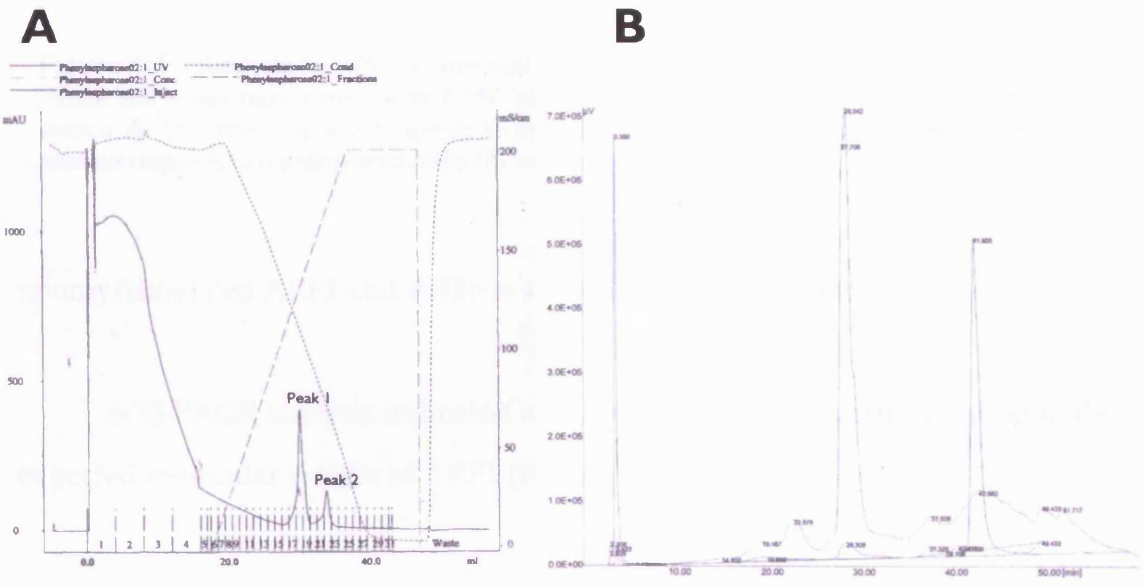


Figure 4.2 **Bovine ARF1 was coexpressed with NMT and purified as described in Materials and Methods section.**

A. *Elution of myristoylated recombinant ARF1 from phenyl-Superose.* Fractions pooled from the Ni-NTA column were concentrated and made to 1 M with solid ammonium sulphate and applied to the phenyl-Superose column equilibrated with buffer containing 1 M ammonium sulphate. Proteins were eluted with a descending ammonium sulphate gradient and 8 ml fractions were collected. Protein elution was monitored by absorbance at 280 nm. The peaks of nonmyristoylated (peak 1) and myristoylated (peak 2) are indicated.

B. *Analysis by HPLC of purified recombinant bovine ARF1.* The two peaks correspond to nonmyristoylated ARF1 ($R_T \approx 28$ min) and 100% myristoylated ARF1 ($R_T \approx 41$ min). The peaks can be seen to have been resolved in the two separate, but overlaid, chromatograms.

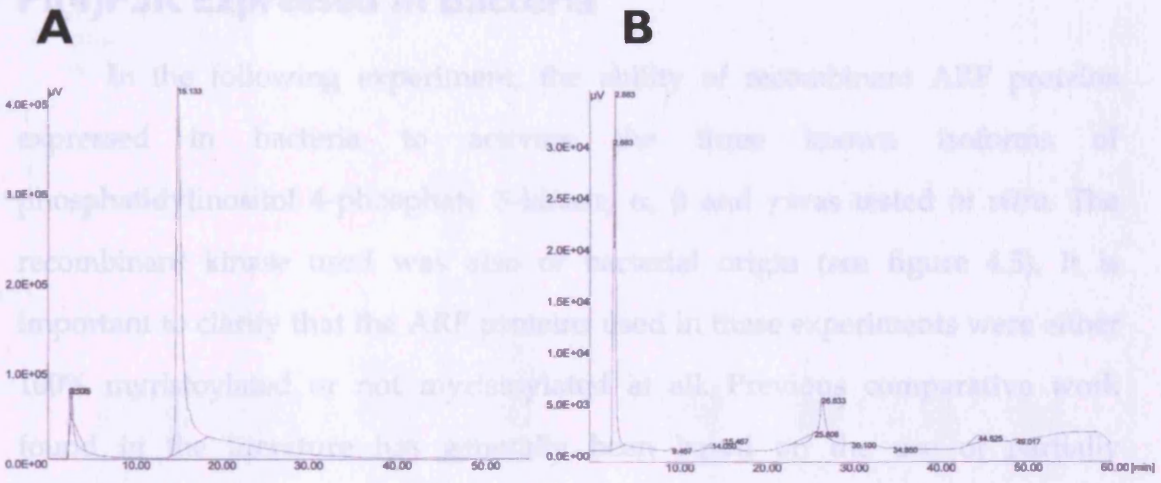


Figure 4.3 Analysis by HPLC of purified mouse ARF6.
Mouse ARF6 was coexpressed with NMT and purified as described in Materials and Methods section. **A.** The second peak corresponds to nonmyristoylated ARF6 ($R_T \approx 15$ min). **B.** The second peak corresponds to myristoylated ARF6 ($R_T \approx 26$ min).

nonmyristoylated ARF1 and ARF6 is shown in Figures 4.2 and 4.3.

SDS-PAGE analysis indicated a unique 21 kDa band corresponding to the expected molecular weight of ARF1 (Figure 4.4).

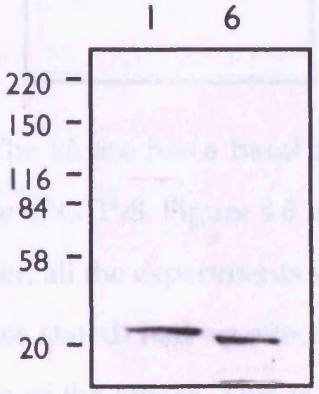


Figure 4.4 SDS-PAGE analysis of purified recombinant myristoylated ARF1 and ARF6.
SDS-PAGE analysis of fractions of His-tagged myristoylated ARF1 and ARF6 following the hydrophobic interaction chromatographic step. The proteins were visualised using Coomassie blue dye.

ARF Proteins Activate All Isoforms Of Recombinant Type I PI(4)P5K Expressed In Bacteria

In the following experiment, the ability of recombinant ARF proteins expressed in bacteria to activate the three known isoforms of phosphatidylinositol 4-phosphate 5-kinase, α, β and γ was tested *in vitro*. The recombinant kinase used was also of bacterial origin (see figure 4.5). It is important to clarify that the ARF proteins used in these experiments were either 100% myristoylated or not myristoylated at all. Previous comparative work found in the literature has generally been based on the use of partially myristoylated recombinant ARF proteins, with no exact details about the degree of myristoylation or equalisation of this factor between ARF isoforms. This is a key point when it comes to comparing the potency of activation of the lipid kinase by different ARFs, especially if it is true that the myristoylation has an effect on the interaction.

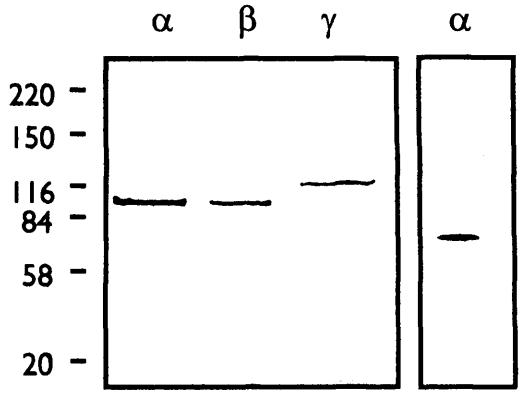


Figure 4.5 SDS-PAGE analysis of purified recombinant Type I PI(4)P5Ks. SDS-PAGE analysis of fractions of recombinant GST-tagged PI(4)P5K α, β and γ expressed in bacteria (left panel), and recombinant HA-tagged PI(4)P5Kα (right panel). The proteins were visualised using Coomassie blue dye.

The kinase has a basal activity and this can be increased by ARFs in the presence of GTPγS. Figure 4.6 shows that 100% myristoylated ARF1 used at 1 μM (hereafter, all the experiments were performed using a 1 μM concentration unless otherwise stated) had an effect in the amount of $PI(4,5)P_2$ generated by all three isoforms of the kinase. This is the first demonstration of the universality of this

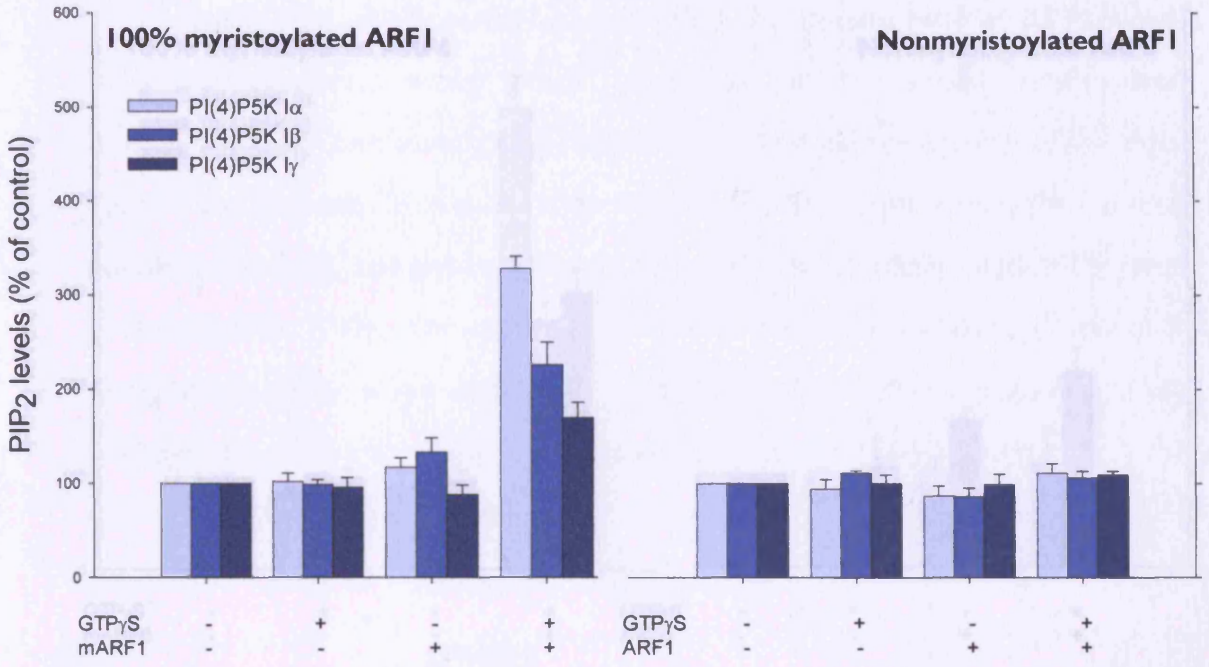


Figure 4.6 ***In vitro* activation of recombinant Type I PI(4)P5K α, β and γ expressed in bacteria by 100% myristoylated ARF1 and nonmyristoylated ARF1.**
Recombinant Type I PI(4)P5K was expressed in *E. coli* as GST-tagged fusion protein, recovered on glutathione-sepharose beads, eluted from the beads with 25 mM reduced glutathione, dialysed and stored in frozen aliquots. A sample of each kinase was taken and diluted in kinase buffer to obtain detectable and similar phosphorylation levels, and mixed with the different ARFs at 1 μM final concentration with or without 10 μM GTPγS. Reactions were stopped after 20 minutes at 25°C by addition of 80 μl of 10% (v/v) HCl. PIP_2 levels were measured as described in the Materials and Methods section.
Data shown are mean ± S.E.M. of 4 (mARF1) and 3 (ARF1) separate experiments within which measurements were made in triplicate. For comparison, data are shown relative to the appropriate internal control since the absolute activity varied with kinase isoform. The statistical significance of the data was evaluated using Student's *t* test.

effect towards all known PI(4)P5K isoforms. The effect was more prominent in the case of the α isoform, 328 ± 13%, followed by PI(4)P5K β, 226 ± 24% and γ, 170 ± 16% ($P \leq 0.004/n=4$). In all cases, the sole addition of GTPγS or the recombinant protein in the absence of GTPγS was not sufficient to increase the production of $PI(4,5)P_2$. When the same experiment was performed using nonmyristoylated ARF1, there was no variation on the activity of the kinase in comparison to the control in any of the three isoforms assayed. This means that the myristate group is strictly necessary for the activation of PI(4)P5K by ARF1.

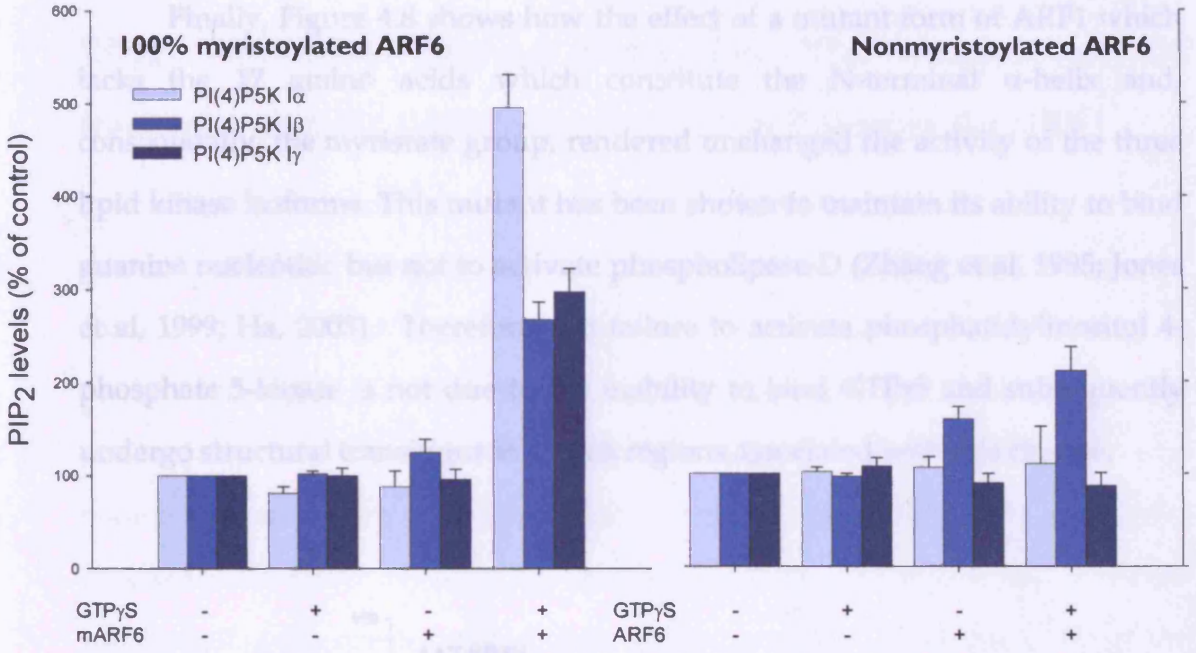


Figure 4.7 ***In vitro* activation of recombinant Type I PI(4)P5K α, β and γ expressed in bacteria by 100% myristoylated ARF6 and nonmyristoylated ARF6.**
Recombinant Type I PI(4)P5K was expressed in *E. coli* as GST-tagged fusion protein, recovered on glutathione-sepharose beads, eluted from the beads with 25 mM reduced glutathione, dialysed and stored in frozen aliquots. A sample of each kinase was taken and diluted in kinase buffer to obtain detectable and similar phosphorylation levels, and mixed with the different ARFs at 1 μM final concentration with or without 10 μM GTPγS. Reactions were stopped after 20 minutes at 25°C by addition of 80 μl of 10% (v/v) HCl. PIP_2 levels were measured as described in the Materials and Methods section.
Data shown are mean ± S.E.M. of 4 separate experiments within which measurements were made in triplicate. For comparison, data are shown relative to the appropriate internal control since the absolute activity varied with kinase isoform. The statistical significance of the data was evaluated using Student's *t* test.

The requirement for myristoylation was also observed when ARF6 was used instead of ARF1 (Figure 4.7). In this case, 100% myristoylated ARF6 activated the α isoform more strongly, 496 ± 36%, followed by γ and then β, 298 ± 25% and 268 ± 19% ($p \leq 0.001/n=3$) respectively. Curiously, there was also a small effect of mARF6 in the absence of GTPγS in the case of the murine β isoform (125 ± 14%) ($p=0.019/n=3$). In the presence of non myristoylated ARF6, neither the α nor the γ isoforms were activated. However, the levels of PIP_2 generated by the β isoform were still increased in the absence of the myristate group (203 ± 34%), and even in the absence of GTPγS (159 ± 13%) ($P \leq 0.012/n=3$).

Finally, Figure 4.8 shows how the effect of a mutant form of ARF1 which lacks the 17 amino acids which constitute the N-terminal α-helix and, consequently, the myristate group, rendered unchanged the activity of the three lipid kinase isoforms. This mutant has been shown to maintain its ability to bind guanine nucleotide but not to activate phospholipase D (Zhang et al, 1995; Jones et al, 1999; Ha, 2003). Therefore, the failure to activate phosphatidylinositol 4-phosphate 5-kinase is not due to the inability to bind GTPγS and subsequently undergo structural transitions in switch regions associated with this change.

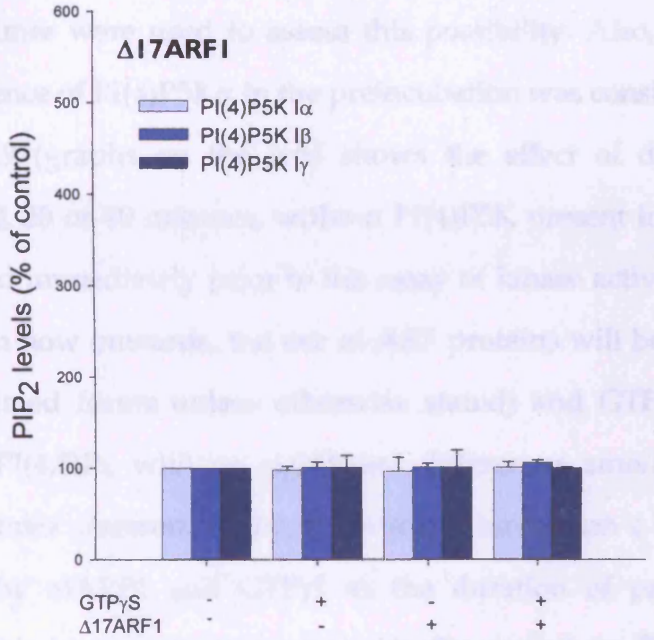


Figure 4.8 ***In vitro* activation of recombinant Type I PI(4)P5K α, β and γ expressed in bacteria by the nonmyristoylated Δ17ARF1 mutant.**

Recombinant Type I PI(4)P5K was expressed in *E. coli* as GST-tagged fusion protein, recovered on glutathione-sepharose beads, eluted from the beads with 25 mM reduced glutathione, dialysed and stored in frozen aliquots. A sample of each kinase was taken and diluted in kinase buffer to obtain detectable and similar phosphorylation levels, and mixed with the different ARFs at 1 μM final concentration with or without 10 μM GTPγS. Reactions were stopped after 20 minutes at 25°C by addition of 80 μl of 10% (v/v) HCl. PIP_2 levels were measured as described in the Materials and Methods section.

Data shown are mean ± S.E.M. of 3 separate experiments within which measurements were made in triplicate. For comparison, data are shown relative to the appropriate internal control since the absolute activity varied with kinase isoform. The statistical significance of the data was evaluated using Student's *t* test.

Effect Of The Temperature On The Activation Of Recombinant PI(4)P5Kα Expressed In Bacteria By ARF1

The previous experiments were performed by preincubating the reaction mixture containing the lipid kinase, ARF, GTPγS and PI(4)P:DMPC vesicles in the absence of ATP at 37°C for 40 minutes. This is to maximise the exchange of nucleotides on ARFs and to allow protein-complex formation. Subsequently, the mix was set at room temperature and [γ-^{32}P]ATP together with carrier ATP was added to start the phosphorylation reaction. But considering that this preincubation could be altering the properties of the interaction, different preincubation times were used to assess this possibility. Also, the effect of the presence or absence of PI(4)P5Kα in the preincubation was considered.

Figure 4.9 (graphs on the left) shows the effect of different times of preincubation, 0, 20 or 40 minutes, without PI(4)P5K present in the mixture but added at the end immediately prior to the assay of kinase activity. The presence of mARF1 (from now onwards, the use of ARF proteins will be restricted to the 100% myristoylated forms unless otherwise stated) and GTPγS increased the production of PI(4,5)P_2, with no significant differences amongst the different preincubation times assessed. If any, there might have been a slight increase in the activation by mARF1 and GTPγS as the duration of preincubation was increased. But this improvement is relative to the control condition where there was only phosphatidylinositol 4-phosphate 5-kinase α and PI(4)P vesicles and no preincubation. The activity of the control was also augmented by simply preincubating the reaction mixture, which means that in terms relative to each internal control, there was not only no improvement but a decrease in the effect of mARF1.

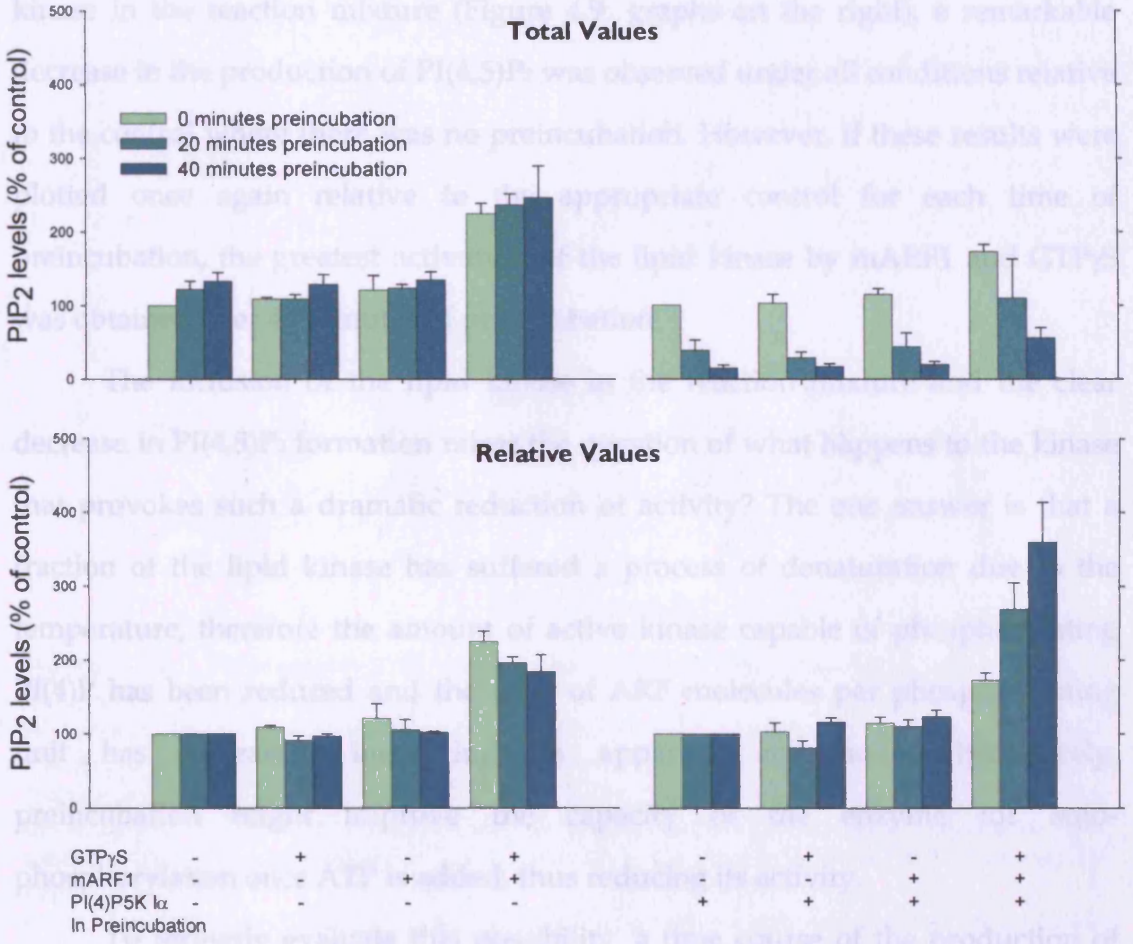


Figure 4.9 ***In vitro* activation of recombinant PI(4)P5K Iα expressed in bacteria by mARF1 after different times of preincubation with and without the kinase.**

Recombinant Type I PI(4)P5Kα was expressed in *E. coli* as GST-tagged fusion protein, recovered on glutathione-sepharose beads, eluted from the beads with 10 mM reduced glutathione, dialysed and stored in frozen aliquots. A sample of the kinase was taken and diluted in kinase buffer to obtain detectable phosphorylation levels. PI(4)P/DMPC vesicles were mixed with or without recombinant mARF1 at 1 μM final concentration and with or without 10 μM GTPγS. The mixtures were incubated at 37°C with or without the lipid kinase for 0, 20 or 40 minutes, after which they were set at room temperature and 50 μM MgATP/[γ-^{32}P]ATP final concentration were added to start the phosphorylation reaction. After 20 minutes at 25°C, the reaction was quenched by addition of 80 μl of 10% (v/v) HCl. PIP_2 levels were measured as described in the Materials and Methods section.

Data shown are mean ± S.E.M. of 3 separate experiments within which measurements were made in triplicate. For comparison, data have been plotted using the absolute values in relation to the control with only lipid vesicles (Total Values), and relative to the appropriate internal control since activity of the kinase varied with the different preincubation times (Relative Values). The statistical significance of the data was evaluated using Student's *t* test.

When the experiment was repeated but now with the inclusion of the lipid kinase in the reaction mixture (Figure 4.9, graphs on the right), a remarkable decrease in the production of $PI(4,5)P_2$ was observed under all conditions relative to the control where there was no preincubation. However, if these results were plotted once again relative to the appropriate control for each time of preincubation, the greatest activation of the lipid kinase by mARF1 and GTPγS was obtained after 40 minutes of preincubation.

The inclusion of the lipid kinase in the reaction mixture and the clear decrease in $PI(4,5)P_2$ formation raises the question of what happens to the kinase that provokes such a dramatic reduction of activity? The one answer is that a fraction of the lipid kinase has suffered a process of denaturation due to the temperature, therefore the amount of active kinase capable of phosphorylating PI(4)P has been reduced and the ratio of ARF molecules per phosphorylating unit has increased, improving its apparent activation. Alternatively, preincubation might improve the capacity of the enzyme for auto-phosphorylation once ATP is added, thus reducing its activity.

To properly evaluate this possibility, a time course of the production of $PI(4,5)P_2$ at different temperatures was performed using recombinant phosphatidylinositol 4-phosphate 5-kinase Iα expressed in *E. coli*. As seen in Figure 4.10, the production of $PI(4,5)P_2$ was nearly linear and increased with time when the phosphorylation reaction was carried out at room temperature (25°C). Surprisingly, increasing the temperature provoked a decrease in the amount of $PI(4,5)P_2$ generated, instead of accelerating the phosphorylation of substrate as would be expected from a situation closer to physiological conditions. When the reaction was performed at 30°C, after 20 minutes the amount of product was reduced to around half of the amount of product generated at room temperature ($P=0.04/n=3$). After 40 minutes the reduction was similar, just over one half

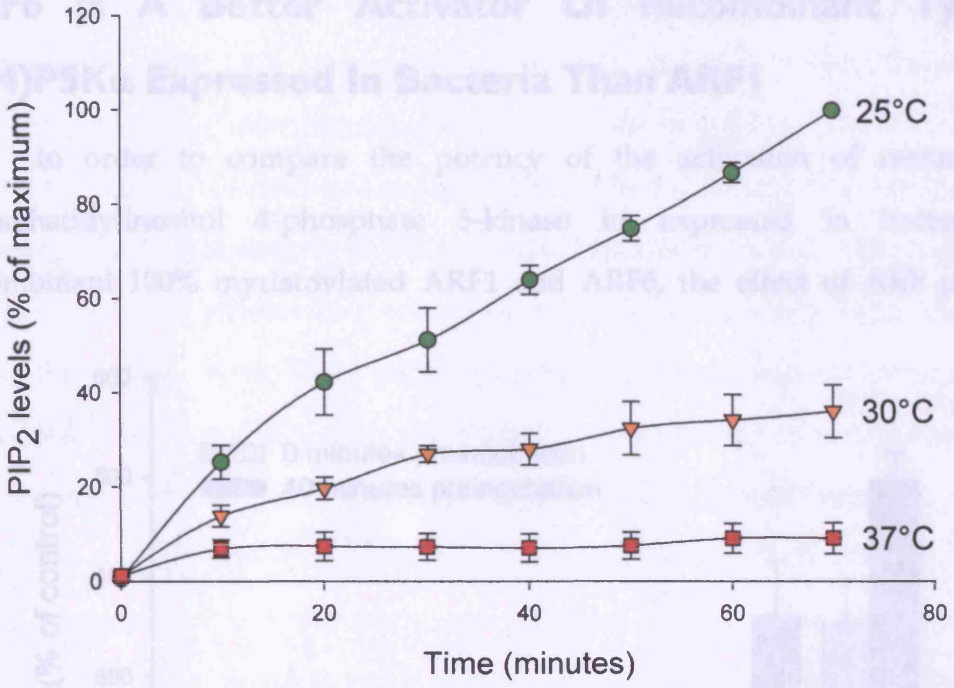


Figure 4.10 Time course of the production of PI(4,5)P_2 by PI(4)P5K Iα expressed in *E. coli* depending on the temperature.

A sample of kinase was taken and diluted in kinase buffer to obtain detectable phosphorylation levels and mixed with PI(4)P/DMPC vesicles. It was then set at the determined temperature, and the reaction was started by addition of MgATP/[γ-^{32}P]ATP. Reactions were sampled at each time point by removal of 3x50 µl identical triplicate samples of each reaction mixture and transferred into new tubes containing 80 µl of 10% (v/v) HCl. PIP_2 levels were measured as described in the Materials and Methods section.

Data shown are mean ± S.E.M. of 3 separate experiments within which measurements were made in triplicate. Data shown have been plotted relative to the maximum overall value. The statistical significance of the data was evaluated using Student's *t* test.

(P=0.003/n=3). When the reaction was performed at 37°C, after 20 minutes the amount of PIP_2 was down to almost 1/6 of the equivalent at 25°C, and after 40 minutes down to 1/9 (P≤0.01/n=3). In fact, the amount of PI(4,5)P_2 generated at 37°C did not increase with time after the first 10 minutes, staying at a constant level throughout the rest of the time course. Clearly the enzyme had been effectively inhibited after only 10 minutes.

ARF6 Is A Better Activator Of Recombinant Type I PI(4)P5Kα Expressed In Bacteria Than ARF1

In order to compare the potency of the activation of recombinant phosphatidylinositol 4-phosphate 5-kinase Iα expressed in bacteria by recombinant 100% myristoylated ARF1 and ARF6, the effect of ARF proteins

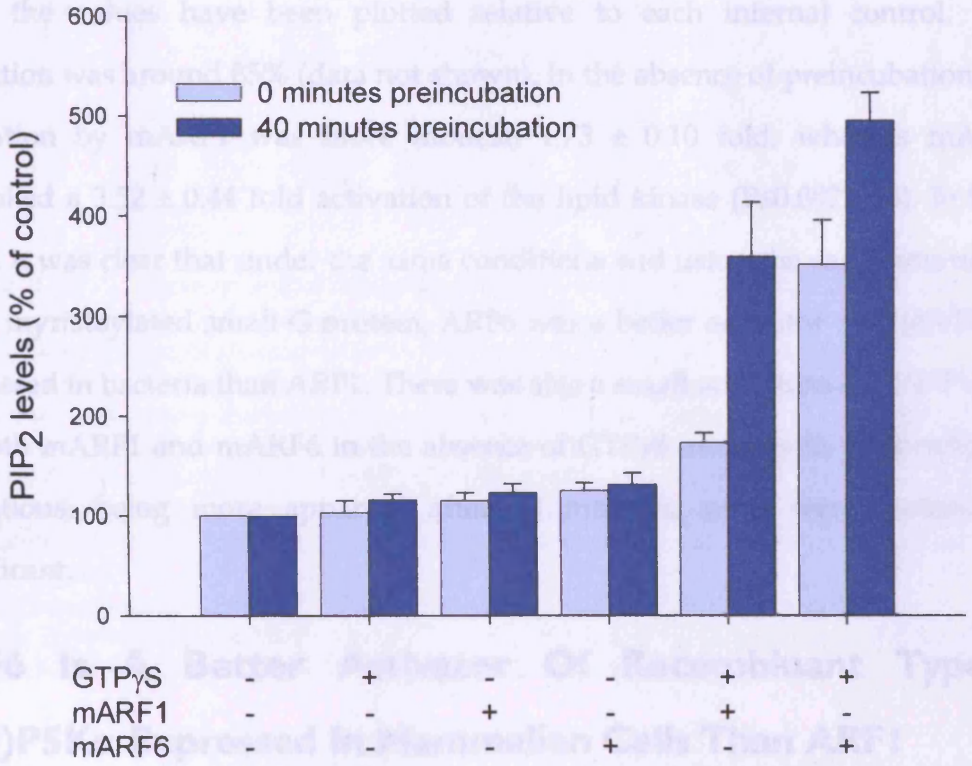


Figure 4.11 ***In vitro* activation of recombinant PI(4)P5K Iα expressed in bacteria by mARF1 and mARF6 after 0 and 40 minutes preincubation.**

A sample of the kinase was taken and diluted in kinase buffer to obtain detectable phosphorylation levels. PI(4)P/DMPC vesicles were mixed with or without recombinant mARF1 and mARF6 at 1 μM final concentration and with or without 10 μM GTPγS. The mixtures were incubated at 37°C with the lipid kinase for 0 or 40 minutes, after which they were set at room temperature and 50 μM MgATP/[γ-^{32}P]ATP final concentration was added to start the phosphorylation reaction. After 20 minutes at 25°C, the reaction was quenched by addition of 80 μl of 10% (v/v) HCl. PIP_2 levels were measured as described in the Materials and Methods section.

Data shown are mean ± S.E.M. of 3 separate experiments within which measurements were made in triplicate. For comparison, data have been plotted relative to the appropriate internal control since activity of the kinase varied with the different preincubation times. The statistical significance of the data was evaluated using Student's *t* test.

after 0 and 40 minutes of preincubation were compared. As Figure 4.11 shows, both mARF1 and mARF6 were good activators of the lipid kinase in the presence of GTPγS, especially after preincubation at 37°C for 40 minutes. mARF1 activated the kinase 3.60 ± 0.54 fold and mARF6 did it 4.96 ± 0.28 fold ($p \leq 0.008/n=3$). But once again, the total amount of PIP_2 produced had been considerably reduced in comparison to the absence of preincubation, which is not noticeable in the graph since the values have been plotted relative to each internal control. This reduction was around 85% (data not shown). In the absence of preincubation, the activation by mARF1 was more modest, 1.73 ± 0.10 fold; whereas mARF6 provoked a 3.52 ± 0.44 fold activation of the lipid kinase ($P \leq 0.002/n=3$). In both cases, it was clear that under the same conditions and using the same amount of 100% myristoylated small G protein, ARF6 was a better activator of PI(4)P5K Iα expressed in bacteria than ARF1. There was also a small activation of PI(4)P5K Iα by both mARF1 and mARF6 in the absence of GTPγS under both preincubation conditions, being more apparent after 40 minutes; none were statistically significant.

ARF6 Is A Better Activator Of Recombinant Type I PI(4)P5Kα Expressed In Mammalian Cells Than ARF1

All the previous experiments were performed using recombinant phosphatidylinositol 4-phosphate 5-kinase expressed in bacteria. This raises the question of how trustworthy the results are considering that any post-translational modifications of the enzyme are not achieved by using prokaryotic expression systems. To clarify this matter, the same experiments were carried out using recombinant phosphatidylinositol 4-phosphate 5-kinase α expressed in mammalian cell lines, typically Cos7 cells. The recombinant kinase had a HA tag

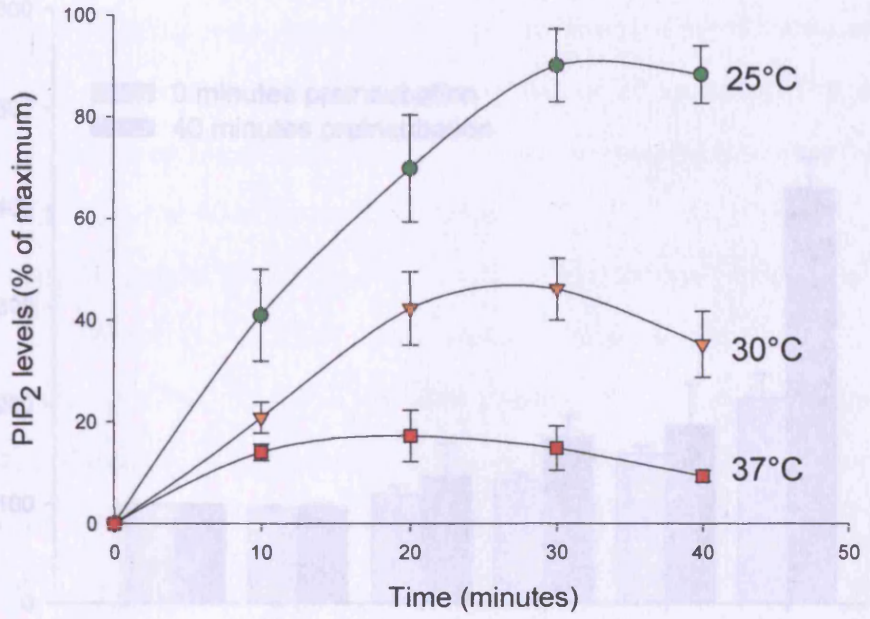


Figure 4.12 Time course of the production of PI(4,5)P_2 by PI(4)P5K Iα expressed in Cos7 cells depending on the temperature.

Recombinant PI(4)P5K Iα was expressed in Cos7 cells as HA-tagged fusion protein, recovered on anti-HA antibody-coated agarose beads, washed and stored in frozen aliquots. A sample of kinase-loaded beads was taken and diluted in kinase buffer to obtain detectable phosphorylation levels and mixed with PI(4)P/DMPC vesicles. It was then set at the determined temperature, and the reaction was started by addition of MgATP/[γ-^{32}P]ATP. Reactions were sampled at each time point by removal of 3x50 μl identical triplicate samples of each reaction mixture and transferred into new tubes containing 80 μl of 10% (v/v) HCl. PIP_2 levels were measured as described in the Materials and Methods section.

Data shown are mean ± S.E.M. of 3 separate experiments within which measurements were made in triplicate. Data shown have been plotted relative to the maximum overall value. The statistical significance of the data was evaluated using Student's *t* test.

fused to its C-terminus which allowed for easy recovery after expression and lysis of the cells, using anti-HA antibody-coated (12CA5) sepharose beads.

Firstly, the influence of the temperature on the lipid kinase expressed in mammalian cells attached to the beads was tested. Once again increasing the temperature reduced the final amount of PI(4,5)P_2 as seen in Figure 4.12. In this case, when the reaction was run at room temperature (25°C), the phosphorylation rate stayed approximately linear only up to 30 minutes, whereas in the case of the enzyme expressed in *E. coli* it was linear all the way up

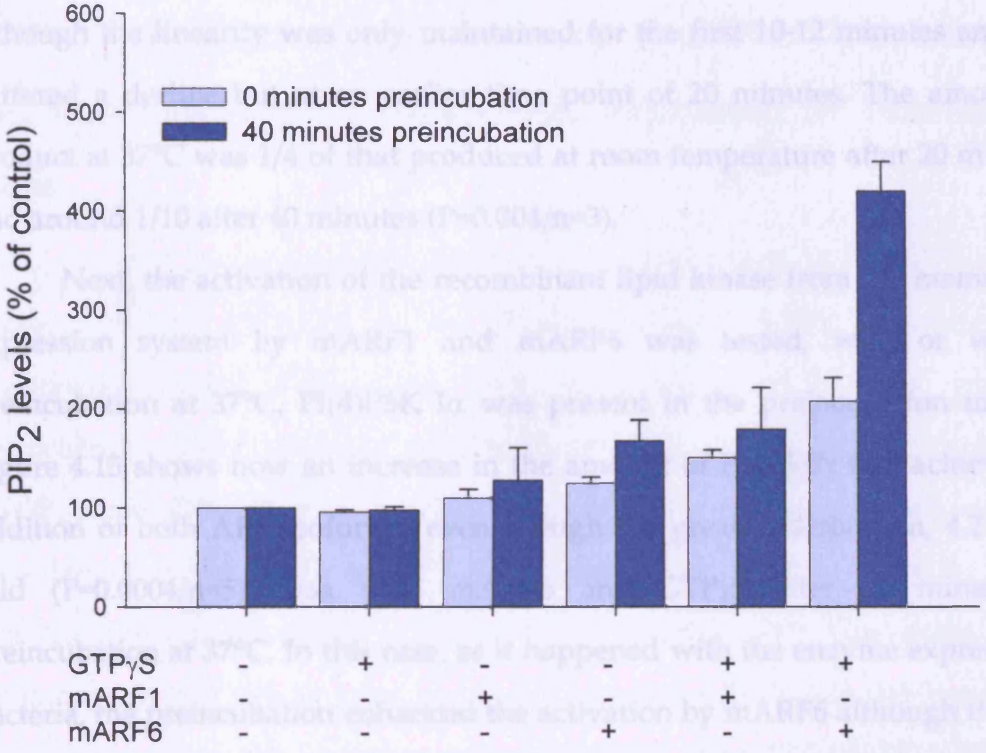


Figure 4.13 ***In vitro* activation of recombinant PI(4)P5K Iα expressed in Cos7 cells by mARF1 and mARF6 after 0 and 40 minutes preincubation.**

A sample of the kinase was taken and diluted in kinase buffer to obtain detectable phosphorylation levels. PI(4)P/DMPC vesicles were mixed with or without recombinant mARF1 and mARF6 at 1 μM final concentration and with or without 10 μM GTPγS. The mixtures were incubated at 37°C with the lipid kinase for 0 or 40 minutes, after which they were set at room temperature and 50 μM MgATP/[γ-^{32}P]ATP final concentration was added to start the phosphorylation reaction. After 20 minutes at 25°C, the reaction was quenched by addition of 80 μl of 10% (v/v) HCl. PIP_2 levels were measured as described in the Materials and Methods section.

Data shown are mean ± S.E.M. of 5 separate experiments within which measurements were made in triplicate. For comparison, data have been plotted relative to the appropriate internal control since activity of the kinase varied with the different preincubation times. The statistical significance of the data was evaluated using Student's *t* test.

to at least 70 minutes (see Figure 4.10). When the reaction was run at 30°C, the amount of product generated was also lower than that generated at 25°C, and it was maintained linear only up until 20 minutes. After that time point, the production of PI(4,5)P_2 did not increase for another 10 minutes and subsequently it declined possibly indicating PI(4,5)P_2 hydrolysis, but this was not explored further. The same pattern was observed when the reaction was run at 37°C,

although the linearity was only maintained for the first 10-12 minutes and also suffered a decline but at an earlier time point of 20 minutes. The amount of product at 37°C was 1/4 of that produced at room temperature after 20 minutes, and around 1/10 after 40 minutes (P=0.004/n=3).

Next, the activation of the recombinant lipid kinase from the mammalian expression system by mARF1 and mARF6 was tested, with or without preincubation at 37°C. PI(4)P5K Iα was present in the preincubation mixture. Figure 4.13 shows how an increase in the amount of $PI(4,5)P_2$ was achieved by addition of both ARF isoforms, even though the greatest activation, 4.21 ± 0.3 fold (P=0.0004/n=5), was with mARF6 and GTPγS after 40 minutes of preincubation at 37°C. In this case, as it happened with the enzyme expressed in bacteria, the preincubation enhanced the activation by mARF6 although the total levels of $PI(4,5)P_2$ were considerably reduced if compared to the levels obtained without preincubation (it cannot be seen in the graph since the data have been plotted relative to the control of each preincubation condition). This reduction was of around 95% of the activity (data not shown). In the absence of preincubation, the activation by mARF6 and GTPγS was also considerable, 2.08 ± 0.24 fold (P=0.011/n=5), even though it was less prominent. Again, there was an effect by mARF6 in the absence of GTPγS, 1.80 ± 0.42 fold (P=0.034/n=5) after preincubating the reaction mix. In the case of mARF1, the effect over the production of $PI(4,5)P_2$ was much less significant than that obtained with the lipid kinase expressed in bacteria. The recombinant small G protein also exerted a positive influence on the activity of PI(4)P5K Iα, 1.51 ± 0.08 fold (P=0.003/n=5), but preincubating the mixture did not improve this effect considerably, 1.8 ± 0.42 fold, as it did with the bacterial kinase or with mammalian mARF6. All in all, ARF6 was a better activator of phosphatidylinositol 4-phosphate 5-kinase Iα in all different conditions tested (after 40 minutes preincubation P=0.009/n=5).

ARF Myristoylation Is Necessary For The Activation Of Recombinant Type I PI(4)P5Kα Expressed In Mammalian Cells

Figure 4.14A presents the effect of nonmyristoylated ARF1, ARF6 and the deletion mutant Δ17ARF1 on the production of $PI(4,5)P_2$ by recombinant phosphatidylinositol 4-phosphate 5-kinase Iα expressed in Cos7 cells. No preincubation was carried out prior to starting the phosphorylation reaction of PI(4)P. None of the three isoforms was capable of activating the lipid kinase to considerable levels at any of the two concentrations assayed, 1 and 10 μM. In fact, the opposite effect was achieved in the case of ARF1. Unexpectedly, the production of $PI(4,5)P_2$ was reduced as the amount of ARF1 was increased. At 10 μM, the amount of PIP_2 had been reduced down to 48 ± 0.13% (P=0.01/n=3) of the control, and slightly more with GTPγS. Δ17ARF1 did now show an inhibitory effect at low concentrations, but it did as the amount of the mutant small G protein was increased to 10 μM. At that concentration, Δ17ARF1 in the presence of GTPγS reduced the lipid kinase activity down to half of the control (P=0.009/n=3). None of these inhibitory effects were seen in the presence of ARF6. It must be pointed out that all three isoforms, specially at 1 μM, seem to provoke a reduction in activity of PI(4)P5K Iα when GTPγS is present in comparison to when its absent. This is quite striking, considering that the activation by small G proteins of the ARF family is dependent on the binding of the nucleotide, or at least when they are myristoylated. Thus the influence of partially myristoylated ARF proteins in activation assays should be carefully reconsidered due to the inverse behaviour of the nonmyristoylated forms at high concentrations, which would be altering the overall picture of activation. Obviously, data obtained by

other investigators using partially myristoylated ARFs will need to be re-evaluated.

Comparatively, Figure 4.14B shows the effect of the myristoylated forms of ARF1 and 6 on $PI(4,5)P_2$ generation in the absence of preincubation. Both

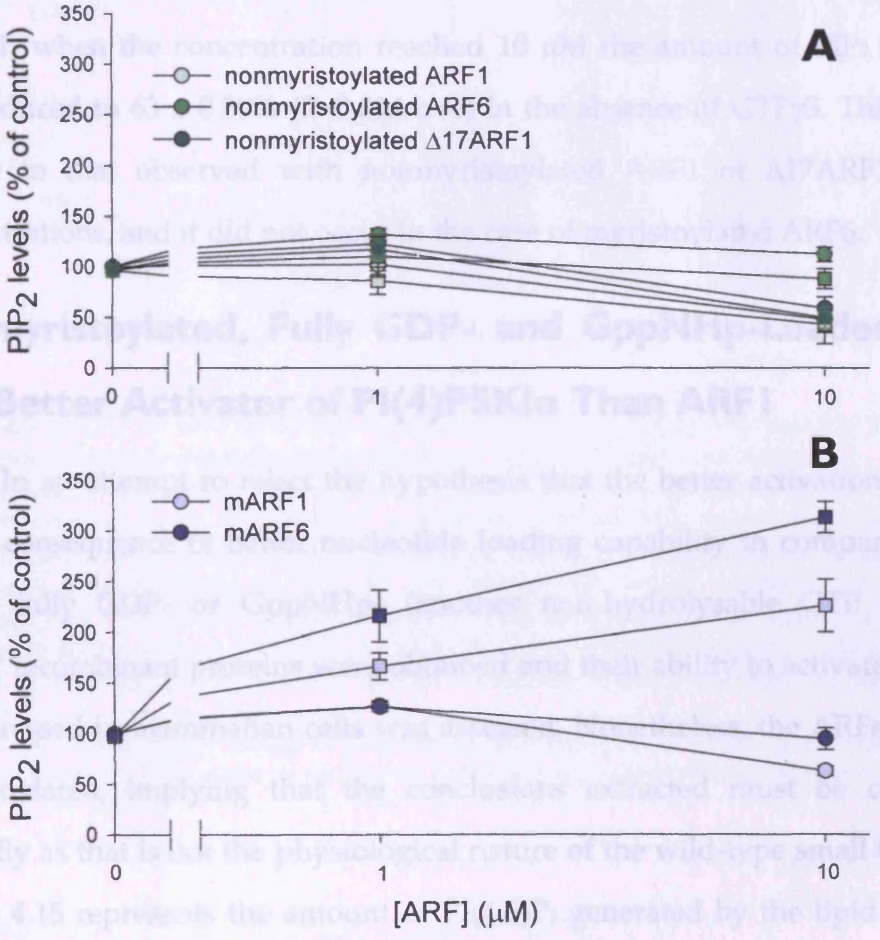


Figure 4.14 ***In vitro*** **activation of recombinant PI(4)P5K Iα expressed in Cos7 cells by different ARFs at different concentrations.**

Recombinant Type I PI(4)P5K α was expressed in Cos7 cells as HA-tagged fusion protein, recovered on anti-HA antibody-coated agarose beads, washed and stored in frozen aliquots. A sample of kinase-loaded beads was taken and diluted in kinase buffer to obtain detectable phosphorylation levels. It was then mixed with PI(4)P/DMPC vesicles, with or without 1 or 10 μM ARF proteins and with (■) or without (●) 10 μM GTPγS at room temperaure. The reaction was started by addition of MgATP/[γ-^{32}P]ATP. After 20 minutes at 25°C, 80 μl of 10% (v/v) HCl were added to quench the reaction. PIP_2 levels were measured as described in the Materials and Methods section.

Data shown are mean ± S.E.M. of 4 (**A**) or 3 (**B**) separate experiments within which measurements were made in triplicate. Data shown have been plotted relative to the control. The statistical significance of the data was evaluated using Student's *t* test.

mARF1 and mARF6 activated PI(4)P5K Iα in a concentration dependent manner and exclusively in the presence of GTPγS. At both 1 and 10 μM concentrations, mARF6 was a better activator of the lipid kinase ($P \leq 0.02/n=4$). In the absence of GTPγS there was a very modest activation at 1 μM, of around 26% ($P<0.001/n=4$) by both isoforms which decreased as the amount of ARF raised. In the case of mARF1, when the concentration reached 10 μM the amount of PIP_2 produced was reduced to 63 ± 0.30% ($P=0.014/n=4$) in the absence of GTPγS. This effect is similar to that observed with nonmyristoylated ARF1 or Δ17ARF1 at high concentrations, and it did not occur in the case of myristoylated ARF6.

Nonmyristoylated, Fully GDP- and GppNHp-Loaded ARF6 Is A Better Activator of PI(4)P5KIα Than ARF1

In an attempt to reject the hypothesis that the better activation by ARF6 was a consequence of better nucleotide loading capability in comparison with ARF1, fully GDP- or GppNHp- (another non-hydrolysable GTP analogue) loaded recombinant proteins were obtained and their ability to activate PI(4)P5K Iα expressed in mammalian cells was assessed. Nonetheless, the ARFs were not myristoylated, implying that the conclusions extracted must be considered carefully as that is not the physiological nature of the wild-type small G protein. Figure 4.15 represents the amount of $PI(4,5)P_2$ generated by the lipid kinase in the presence of the different ARF proteins, demonstrating that the preferential activation by ARF6 is still maintained ($p \leq 0.03/p=3$) even after fully loading of the GTPases with both GDP and GppNHp and in the absence of myristoylation. No effect was seen at 25°C or at 1 μM-ARF concentration (not shown).

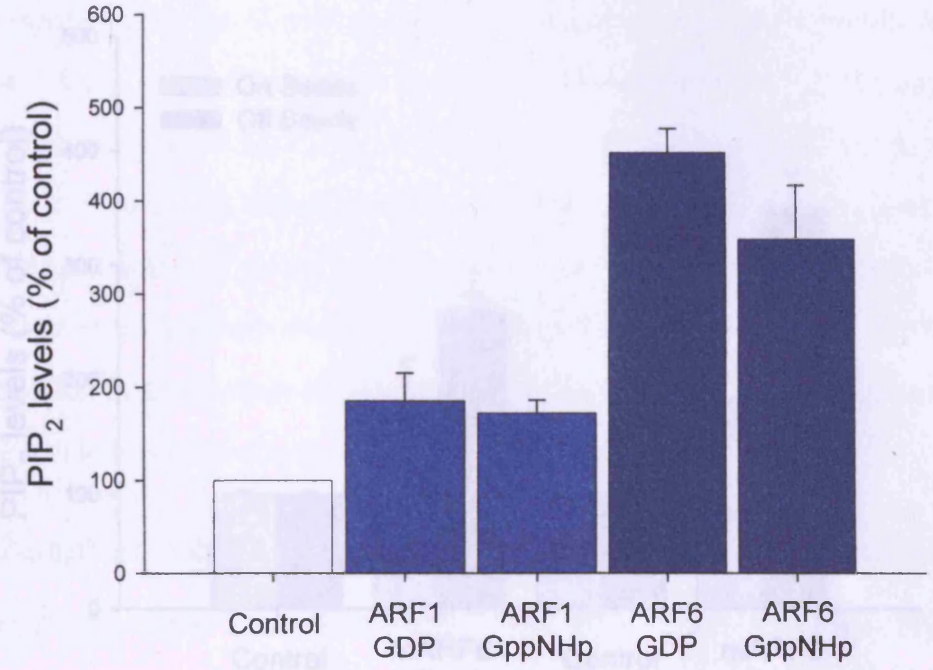


Figure 4.15 ***In vitro*** **activation of recombinant PI(4)P5K Iα expressed in Cos7 cells by recombinant preloaded nonmyristoylated GDP/GTP-ARF1 and ARF6.**
A sample of the kinase was taken and diluted in kinase buffer to obtain detectable phosphorylation levels. PI(4)P/DMPC vesicles were mixed with or without recombinant nonmyristoylated preloaded GppNHp/GDP-ARF1 and ARF6 at 10 μM final concentration. The mixtures were incubated at 37°C for 30 minutes, after which they were set at room temperature and 50 μM MgATP/[γ-^{32}P]ATP final concentration was added to start the phosphorylation reaction. After 20 minutes at 25°C, the reaction was quenched by addition of 80 μl of 10% (v/v) HCl. PIP_2 levels were measured as described in the Materials and Methods section.
Data shown are mean ± S.E.M. of 3 separate experiments within which measurements were made in triplicate. The statistical significance of the data was evaluated using Student's *t* test.

ARF Activates Type I PI(4)P5Kα Eluted From Beads

To rule out the possibility that the apparent activation of Type I phosphatidylinositol 4-phosphate 5-kinase α expressed in a mammalian system by ARF or that its apparent magnitude is an artefactual consequence of differential interaction or release of the lipid kinase from the beads by the small G protein, kinase expressed in mammalian cells was eluted from beads and assayed for activation by mARF6. Since the kinase is bound to the beads by the

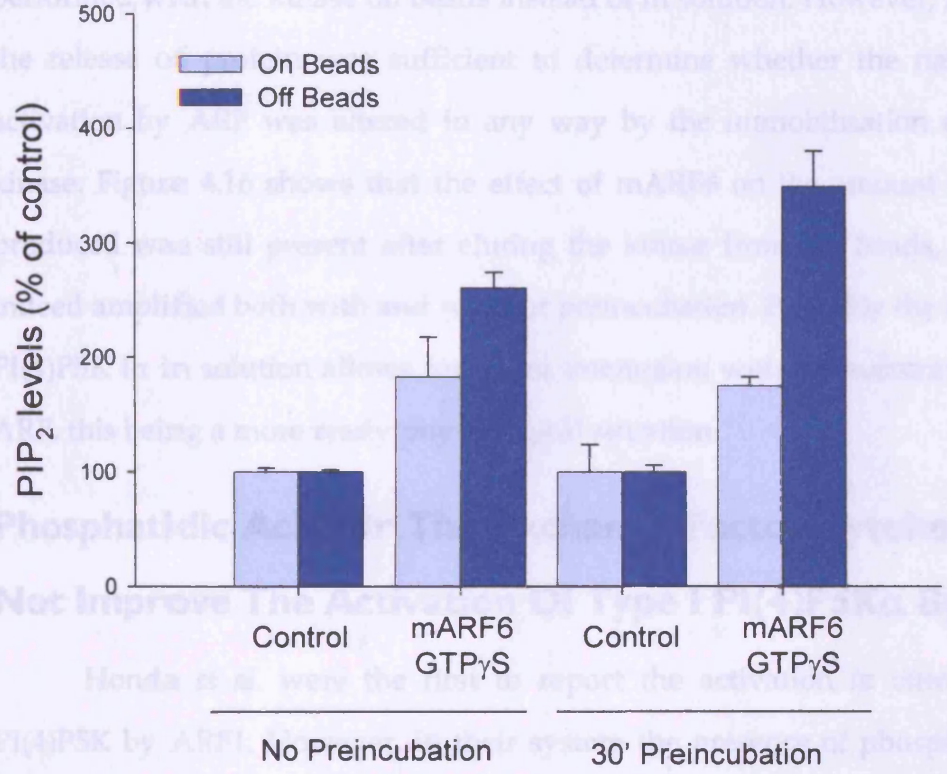


Figure 4.16 ***In vitro* activation of recombinant Type I PI(4)P5K α expressed in Cos7 cells on and off beads by mARF6 after 0 and 30 minutes preincubation.**
Recombinant Type I PI(4)P5K α was expressed in Cos7 cells as HA-tagged fusion protein, recovered on anti-HA antibody-coated agarose beads, washed and stored in frozen aliquots. A sample of the kinase was taken and diluted in kinase buffer to obtain detectable phosphorylation levels. Another sample was used to elute the lipid kinase by addition of 100 µl of 2 mg/ml HA peptide and 2 mg/ml BSA, after removal of the supernatant of the beads. After rotating the beads at 4°C for 1 hour, these were removed by centrifugation and the supernatant used for the experiment. PI(4)P/DMPC vesicles were mixed with or without recombinant ARF6 at 1 µM final concentration and 10 µM GTPγS. The mixtures were incubated at 37°C with the lipid kinase for 0 or 30 minutes, after which they were set at room temperature and 50 µM MgATP/[γ-^{32}P]ATP final concentration was added to start the phosphorylation reaction. After 20 minutes at 25°C, the reaction was quenched by addition of 80 µl of 10% (v/v) HCl. PIP_2 levels were measured as described in the Materials and Methods section.
Data shown are mean ± S.E.M. of triplicate samples within a single experiment. For comparison, data have been plotted relative to the appropriate internal control since activity of the kinase was reduced after the preincubation. The statistical significance of the data was evaluated using Student's *t* test.

HA tag, addition of synthetic HA peptide to the beads releases it into solution, as the peptide competes with the fusion protein to bind to the antibody. The efficiency of this reaction is not very high, giving only small yields of kinase from large amount of beads. This is the reason why all the previous experiments were

performed with the kinase on beads instead of in solution. However, in this case, the release of protein was sufficient to determine whether the nature of the activation by ARF was altered in any way by the immobilisation of the lipid kinase. Figure 4.16 shows that the effect of mARF6 on the amount of $PI(4,5)P_2$ produced was still present after eluting the kinase from the beads, and it was indeed amplified both with and without preincubation. Probably the free state of PI(4)P5K Iα in solution allows for better interaction with the substrate and with ARF, this being a more ready physiological situation.

Phosphatidic Acid Or The Exchange Factor Cytohesin-1 Do Not Improve The Activation Of Type I PI(4)P5Kα By ARF

Honda *et al.* were the first to report the activation *in vitro* of Type I PI(4)P5K by ARF1. However, in their system the presence of phosphatidic acid (PA) was indispensable to achieve the activation of the kinase using PI(4)P/PA vesicles. Later, Jones D.H. *et al.* (2000) found that the reported activation was completely abolished if instead of using PI(4)P vesicles they used a mixture of phosphadylcholine (PC), PI(4)P and PA (10:1:1 ratio respectively). The kinase assay conditions used throughout this thesis include the use of PI(4)P/PC vesicles in a 1:6 ratio (approximately). Figure 4.17 shows how the addition of PA into the lipid vesicles does not improve the activation of PI(4)P5K Iα by ARF proteins. In the case where there is no preincubation, ARF6 activation is slightly reduced by the addition of PA. After preincubation of the samples, the activation is considerably reduced, down to more than half of the control value. PA does not completely abolish the activation by ARF6 in this system as it was reported by Jones D.H. *et al.*, but this is probably due to the different proportions of PA and PC used in the vesicles.

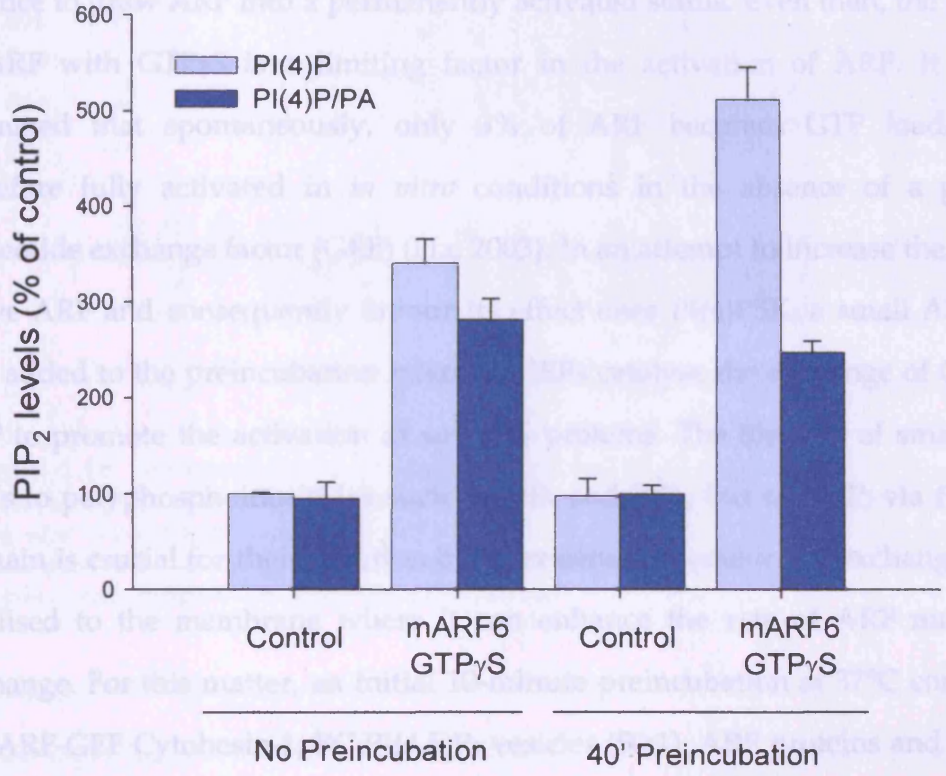


Figure 4.17 ***In vitro*** **activation of recombinant Type I PI(4)P5K α expressed in Cos7 cells with PI(4)P/DMPC or PI(4)P/PA/DMPC vesicles by mARF6 after 0 and 40 minutes preincubation.**

Recombinant Type I PI(4)P5K α was expressed in Cos7 cells as HA-tagged fusion protein, recovered on anti-HA antibody-coated agarose beads, washed and stored in frozen aliquots. A sample of the kinase was taken and diluted in kinase buffer to obtain detectable phosphorylation levels. PI(4)P/DMPC (1:10) or PI(4)P/PA/DMPC (1:1:10) vesicles were mixed with or without recombinant ARF6 at 1 μM final concentration and 10 μM GTPγS. The mixtures were incubated at 37°C with the lipid kinase for 0 or 40 minutes, after which they were set at room temperature and 50 μM MgATP/[γ-^{32}P]ATP final concentration was added to start the phosphorylation reaction. After 20 minutes at 25°C, the reaction was quenched by addition of 80 μl of 10% (v/v) HCl. PIP_2 levels were measured as described in the Materials and Methods section.

Data shown are mean ± S.E.M. of quadruplicate samples within a single experiment. For comparison, data have been plotted relative to the appropriate internal control since activity of the kinase was reduced after the preincubation. The statistical significance of the data was evaluated using Student's *t* test.

Small G proteins are enzymes which depend on the binding of the nucleotide GTP for their activation. GDP-bound ARF is thought to be the biologically inactive state and the substitution of GDP by GTP triggers its activation. The use of the non-hydrolysable compound GTPγS is a widespread

practice to draw ARF into a permanently activated status. Even then, the loading of ARF with GTPγS is a limiting factor in the activation of ARF. It can be estimated that spontaneously, only 3% of ARF becomes GTP loaded and therefore fully activated in *in vitro* conditions in the absence of a guanine nucleotide exchange factor (GEF) (Ha, 2003). In an attempt to increase the ratio of active ARF and consequently favour its effect onto PI(4)P5K, a small ARF-GEF was added to the preincubation mixture. GEFs catalyse the exchange of GDP for GTP to promote the activation of small G proteins. The binding of small ARF-GEFs to polyphosphoinositides such as PIP_2 and PIP_3, but not PIP, via their PH domain is crucial for their function by increasing the amount of exchange factor localised to the membrane where it can enhance the rate of ARF nucleotide exchange. For this matter, an initial 10-minute preincubation at 37°C containing the ARF-GEF Cytohesin-1, PC:$PI(4,5)P_2$ vesicles (50:1), ARF proteins and GDP or GTP was performed, followed by the addition of PI(4)P5Kα, MgATP and its substrate, PI(4)P. Nonetheless, no improvement on PI(4)P5K activation was achieved as compared to conditions where the exchange factor was absent (data not shown). This is probably due to the conditions of the preincubation. Amongst other things, the inclusion of PIP_2 in the assay could be counterproductive as it acts as a product inhibitor of PI(4)P5K (Moritz *et al.*, 1992).

ARF6 Is A Better Promoter Of $PI(4,5)P_2$ Synthesis In Permeabilised HL60 Cells Than ARF1

There have been several reports in the literature showing that ARF proteins promote the synthesis of $PI(4,5)P_2$ in cells. This increase in PIP_2 seems to be by the sole direct activation of Type I phosphatidylinositol 4-phosphate 5-kinase by ARF (Godi *et al.*, 1999; Jones D.H. *et al.*, 2000; Lawrence *et al.*, 2003) or

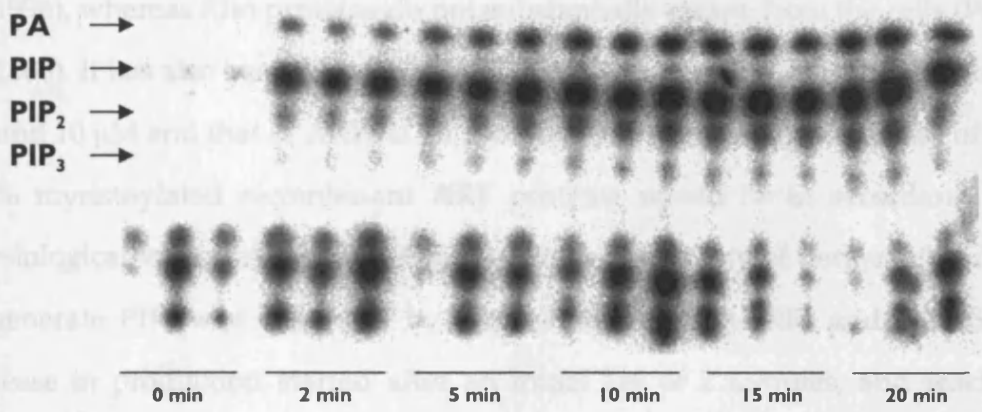


Figure 4.18 Visualisation of [γ-^{32}P]-labelled lipid-content of HL60 cells on TLC plate. Radioactive lipid content of triplicate time points of a typical HL60-cell permeabilisation/reconstitution assay after readdition of recombinant ARF1 and GTPγS (in this particular case). The lipids were separated using the solvent system described in the Materials and Methods section. The migration position of each phosphoinositide is indicated on the left.

in concert with PLD (Fensome *et al.*, 1996; Martin, *et al.*, 1996; Honda, *et al.*, 1999; O'Luanaigh, *et al.*, 2002; Skippen *et al.*, 2002). PLD hydrolyses PC to produce PA which is a potential cellular activator of Type I PI(4)P5K (Moritz *et al.*, 1992; Jenkins *et al.*, 1994; Jones D.R. *et al.*, 2000). In some of these reports, the use of ARF1 and ARF6 has been stated to be equivalent in its potency to promote PIP_2 synthesis (Way *et al.*, 2000; Skippen *et al.*, 2002; O'Luannaigh *et al.*, 2002) but this statement was not based on a rigorous comparison of equally fractionally myristoylated recombinant ARF proteins. Indeed partially myristoylated proteins were used and the degrees of derivatisation were not estimated.

In the following experiment, the effect of readdition of 100% myristoylated ARF1 and ARF6 to permeabilised HL60 cells was compared. Using thin layer chromatographic plates (TLC), variations in the levels of the resolved radiolabelled phospholipids/polyphosphoinositides could be quantified, as depicted on Figure 4.18. It has previously been proven that the majority of ARF proteins leak out of the cells within 5-10 minutes of permeabilisation (Fensome *et*

al., 1996), whereas Rho proteins do not substantially escape from the cells (Way *et al.*, 2000). It has also been reported that the concentration of ARF1 in HL60 cells is around 10 μM and that of ARF6 0.5-1 μM (Skippen *et al.*, 2002), so the use of 1 μM 100% myristoylated recombinant ARF proteins would be in accordance with physiological values. As seen in Figure 4.19, the capability of permeabilised cells to generate PIP_2 was enhanced by the readdition of mARF1 and GTPγS. The increase in production started after an initial lag of 2 minutes, and reached a maximal 1.73 fold-increase in comparison to the GTPγS-only curve after 20 minutes ($p=0.03/n=7$). In the case of mARF6 and GTPγS, the activation was much more potent. The apparent increase had a faster on-set and the generation of PIP_2 increased as time evolved, ranging from 2-fold increased production after 2 minutes up to a 3-fold increase after 20 minutes ($p=2.2x10^{-5}/n=7$). In the case of the sole addition of GTPγS, there was a not very significant increase of around 1.3-fold between minutes 2 and 15 of the reaction and failed to increase after that time point. In all cases, after 15 minutes the amount of PIP_2 declined, probably due to its degradation by other enzymes, most probably phosphatases, which had not been deliberately inhibited.

From the same experiment, readings on the amount of PIP and PA showed no major changes, except those caused by the addition of GTPγS on its own. Thus, the production of these two metabolites did not seem to be influenced by ARF proteins. The rapid production of radioactive PA could only be a consequence of the hydrolysis of PIP_2 by phospholipase C (PLC) into IP_3 and DAG in this case, and the consequent phosphorylation of DAG by diacylglycerol kinase (DGK). Indeed, historically the accumulation of radiolabelled PA in cells and membranes derived from phosphate transfer from [^{32}P-γ]ATP to DAGs was one of the first definitive experimental indicators of PLC activity. An alternative route for the production of radioactive PA would be by the hydrolysis of PC into

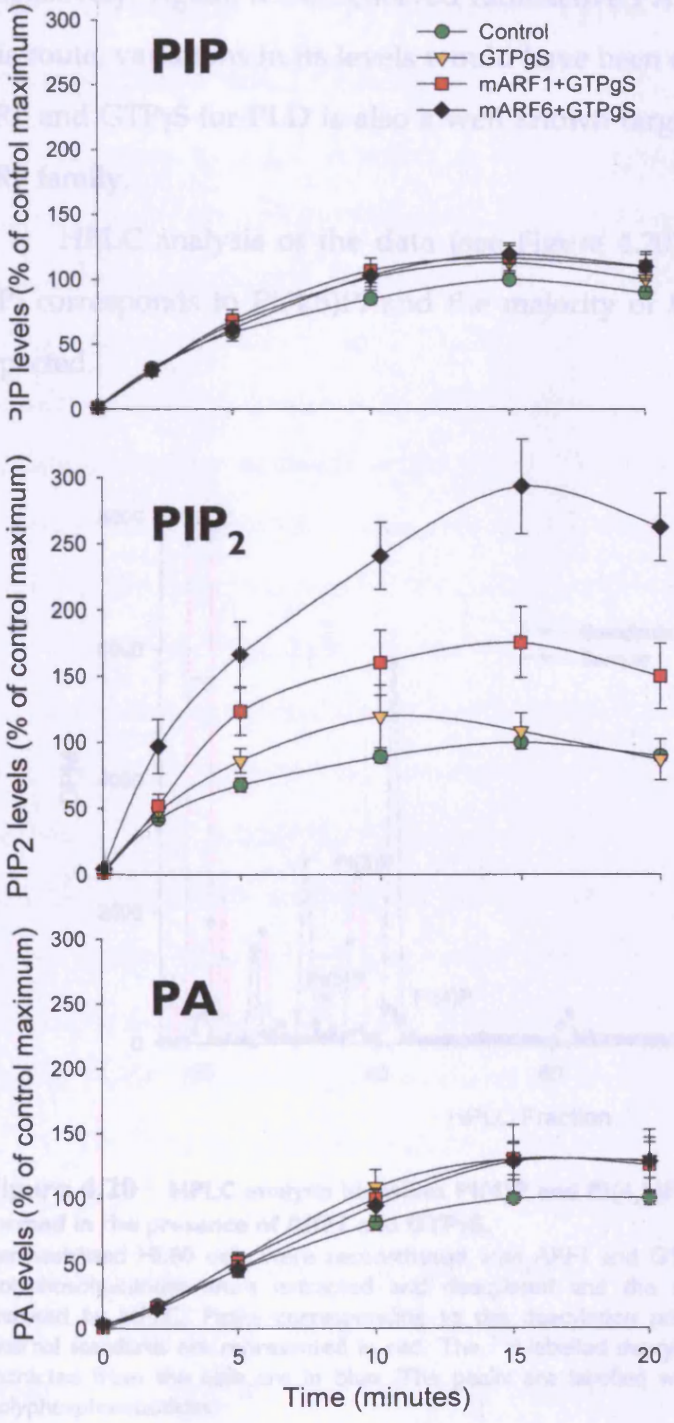


Figure 4.19 **Effect of the readdition of recombinant 100% myristoylated ARF1 or ARF6 and GTPγS on lipid production on permeabilised HL60 cells.**

After 10 minutes of permeabilisation with streptolysin O (0.4 IU/ml) at 37°C, cytosol-depleted HL60 cells were centrifuged and resuspended in buffer containing 50 μM MgATP/[γ-^{32}P]ATP final concentration (added last to start the reaction), 1 μM ARF1 or ARF6 and 10 μM GTPγS. Reactions were run at 37 °C and sampled at each time point by removal of 3x50 μl identical triplicate samples of each reaction mixture and transferred into new tubes containing 80 μl of 10% (v/v) HCl. Lipid levels were measured as described in the Materials and Methods section, using the characteristic migration properties of lipids on a TLC system to quantify the effect of the constituents on the different lipids.

Data shown are mean ± S.E.M. of 7 separate experiments within which measurements were made in triplicate. Data have been plotted relative to the maximum value of the control curve. The statistical significance of the data was evaluated using Student's *t* test.

choline and PA by PLD, and a subsequent dephosphorylation and rephosphorylation cycle by phosphatidate phosphohydrolase (PAP) and DGK

respectively. Again, if the observed radioactive PA had been generated through this route, variations in its levels would have been expected from the addition of ARF and GTPγS for PLD is also a well known target for small G proteins of the ARF family.

HPLC analysis of the data (see Figure 4.20) shows how the majority of PIP_2 corresponds to $PI(4,5)P_2$ and the majority of PIP corresponds to PI(4)P, as expected.

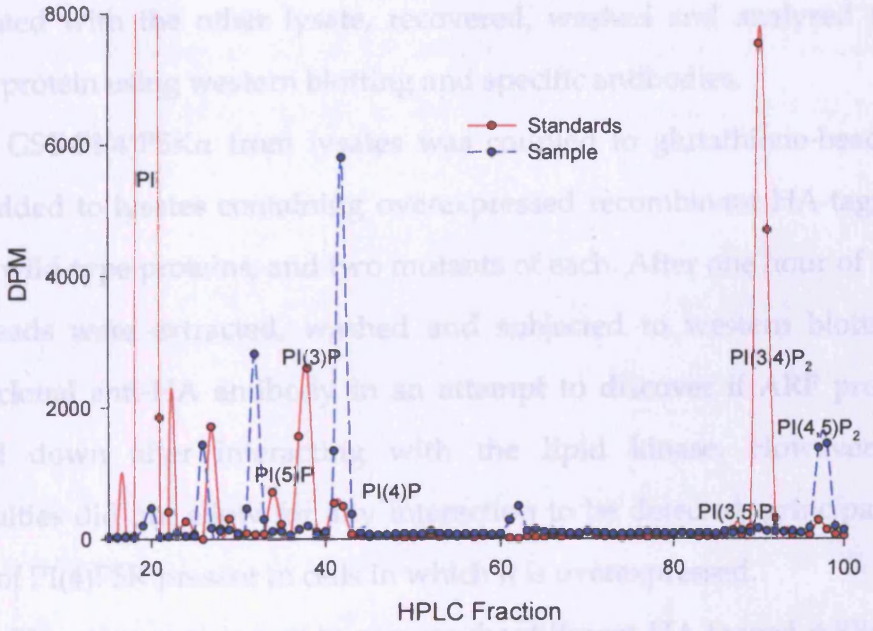


Figure 4.20 HPLC analysis identifies PI(4)P and $PI(4,5)P_2$ as the major phosphoinositides formed in the presence of ARF1 and GTPγS.
Permeabilised HL60 cells were reconstituted with ARF1 and GTPγS, in the presence of [γ^{32}P]ATP. Polyphosphoinitides were extracted and deacylated and the resulting glycerol-phospho inositols analysed by HPLC. Peaks corresponding to the deacylation products of authentic tritium-labelled internal standards are represented in red. The ^{32}P-labelled deacylation products of the inositol lipids extracted from the cells are in blue. The peaks are labelled with respect to the different parent polyphosphoinositides.

ARF Proteins Pull Down Type I PI(4)P5Kα From Transfected HEK293 Cell

In order to asses whether a stable physical interaction between ARF proteins and phosphatidylinositol 4-phosphate 5-kinase α takes place, recombinant HA-tagged ARF proteins and GST-tagged PI(4)P5Kα were overexpressed separately in HEK293 cells. One of the two could then be recovered from the lysates with either HA antibody-coated sepharose beads or glutathione sepharose beads respectively. The beads could then be washed and incubated with the other lysate, recovered, washed and analysed for pulled-down protein using western blotting and specific antibodies.

GST-PI(4)P5Kα from lysates was coupled to glutathione-beads, washed and added to lysates containing overexpressed recombinant HA-tagged ARF 1 and 6 wild-type proteins, and two mutants of each. After one hour of incubation, the beads were extracted, washed and subjected to western blotting with a monoclonal anti-HA antibody in an attempt to discover if ARF proteins were pulled down after interacting with the lipid kinase. However, technical difficulties did not allow for any interaction to be detected, principally the low mass of PI(4)P5K present in cells in which it is overexpressed.

The other option was to recover the different HA-tagged ARFs out of the lysates using HA antibody-coated sepharose beads, and put them in contact with the lysates of overexpressed GST-tagged PI(4)P5Kα. The amount of GST-PI(4)P5Kα recovered bound to ARF was quantified by western blotting using a monospecific anti-peptide PI(4)P5Kα antibody and also by PI 4-phosphate 5-kinase activity assay. In an ideal system, HA antibody-coated beads should not pull down any GST-PI(4)P5Kα since no specific interaction is meant to occur between the two. This is not what happens in reality, and due to the existence of

unspecific interactions between the kinase (amongst other cell proteins) and the beads, some kinase and/or activity was pulled down solely by the beads. Other bead types are known to have fewer non-specific interactions, especially glutathione-beads, but as stated above they did not perform satisfactorily. In an attempt to minimise such interactions, the beads were blocked with bovine serum albumine for 1 hour at 4°C. This procedure clearly reduced the amount of kinase that bound non-specifically to the beads, but it did not completely abolish it. The best solution would have been to swap tags by creating new plasmids with GST-ARF and HA-PI(4)P5Kα to avoid these interactions.

The following model of the interaction was developed to allow for the unspecific binding of the kinase to the beads and therefore be able to detect the specific interaction.

$$\text{Pulled Down Kinase} = \frac{\dfrac{\text{PI(4)P5K I}\alpha}{\text{IgG Light Chain}} - \dfrac{\text{Control}}{\text{IgG Light Chain}}}{\text{ARF}}$$

The factor **Control/IgG Light Chain** normalises for the amount of kinase that is pulled down by unspecific binding, since the mass of beads in each individual sample is equivalent to the amount of anti-HA antibody light chain. The light chain is conveniently quantified by detection with anti-mouse secondary antibody in a later step. Theoretically, the amount of beads and, therefore, the amount of anti-HA light chain should be the same in all cases presuming the same amount has been used to capture the target protein. However, in some of the experiments the amount of beads used for the control and some of the different isoforms were not exactly the same, owing to extensive manipulation of samples during washes, etc (see Figure 4.21). Even in the cases

where seemingly similar amounts of bead slurry had been used, the reading of the light chain showed considerable differences at times. So this is a useful means of normalising the results to account for the final amount of beads recovered and the unspecific binding of the kinase to these beads.

This value is then subtracted to the total amount of kinase detected in the blot, per amount of beads once more, **PI(4)P5K Iα/IgG Light Chain**. This subtraction would then give an idea of the amount of kinase pulled down by specific interaction, since the unspecifically bound kinase has been deducted.

This final figure is divided by the amount of **ARF** detected in the blot using HA antibody in each one of the conditions. This is necessary when it comes to comparing the ability of the different ARF isoforms and mutants to bind the kinase, because the amount of ARF may vary depending on the level of expression in cells in each case. The final number would then be a reliable indicator of the amount of kinase pulled down by specific interaction with ARF.

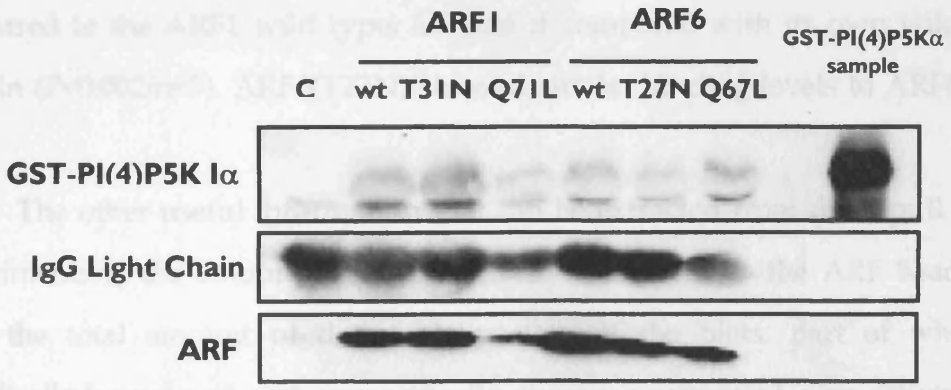


Figure 4.21 **Association of PI(4)P5K Iα with ARF proteins.**
HA-tagged ARF proteins bound to HA antibody-coated beads were incubated with 25 µl of Cos7 cells-lysates from cells transfected with GST-PI(4)P5K Iα. Incubations lasted for 1 hour at 4°C. The beads were washed and the proteins displayed by SDS-PAGE and transferred to a nitrocellulose membrane. The blots were incubated sequentially with anti-PI(4)P5K Iα antibody and anti-HA (anti-recombinant ARF) antibody. The first lane on the left is a control from untransfected cells and the last lane on the right is a sample of recombinant GST-PI(4)P5K Iα produced in bacteria.

As seen in Figure 4.22A, when the beads were loaded with ARF proteins there was an increase in the amount of pulled down PI(4)P5K Iα in comparison to the control. The control shows specific kinase binding but it must be remembered that this is a representation of the detected amount of kinase minus the control. The wild-type proteins and two mutants were used in order to compare whether the activation state of ARF had any influence in the interaction with PI(4)P5K Iα. The GTPase deficient mutants ARF6(Q67L) and ARF1(Q71L) are permanently GTP-bound and so they can be used as constitutively-active ARFs. The GTP-exchange suppressing mutants ARF6(T27N) and ARF1(T31N) are thought to represent permanently GDP bound forms of ARF and are used extensively as dominant-negative mutants. In the case of ARF1, the GTPase deficient ARF1(Q71L) mutant increased the binding 3.4 ± 0.22 fold (P=0.002/n=5) in comparison to the wild type. The GDP-binding ARF1(T31N) mutant did not increase the binding significantly. In the case of ARF6(Q67L), the mutant increased the binding of the lipid kinase by 3.12 ± 0.20 fold (P=0.009/n=5) if compared to the ARF1 wild type, 3.8 fold if compared with its own wild type protein (P=0.002/n=5). ARF6(T27N) showed similar binding levels to ARF6 wild type.

The other useful information that can be extracted from these pull down experiments is the amount of kinase activity associated to the ARF beads. As with the total amount of detectable protein in the blots, part of which is specifically bound and part unspecifically, there is unspecific kinase activity that binds to the beads as well as specific. The previous equation can be used by substituting the protein mass with the amount of $PI(4,5)P_2$ generated in a kinase assay where precipitating beads are added as the source of enzyme. Thus, unspecifically bound kinase activity will be subtracted from the final activity value. Figure 4.22B shows such values of activity. ARF1(Q71L) was the highest,

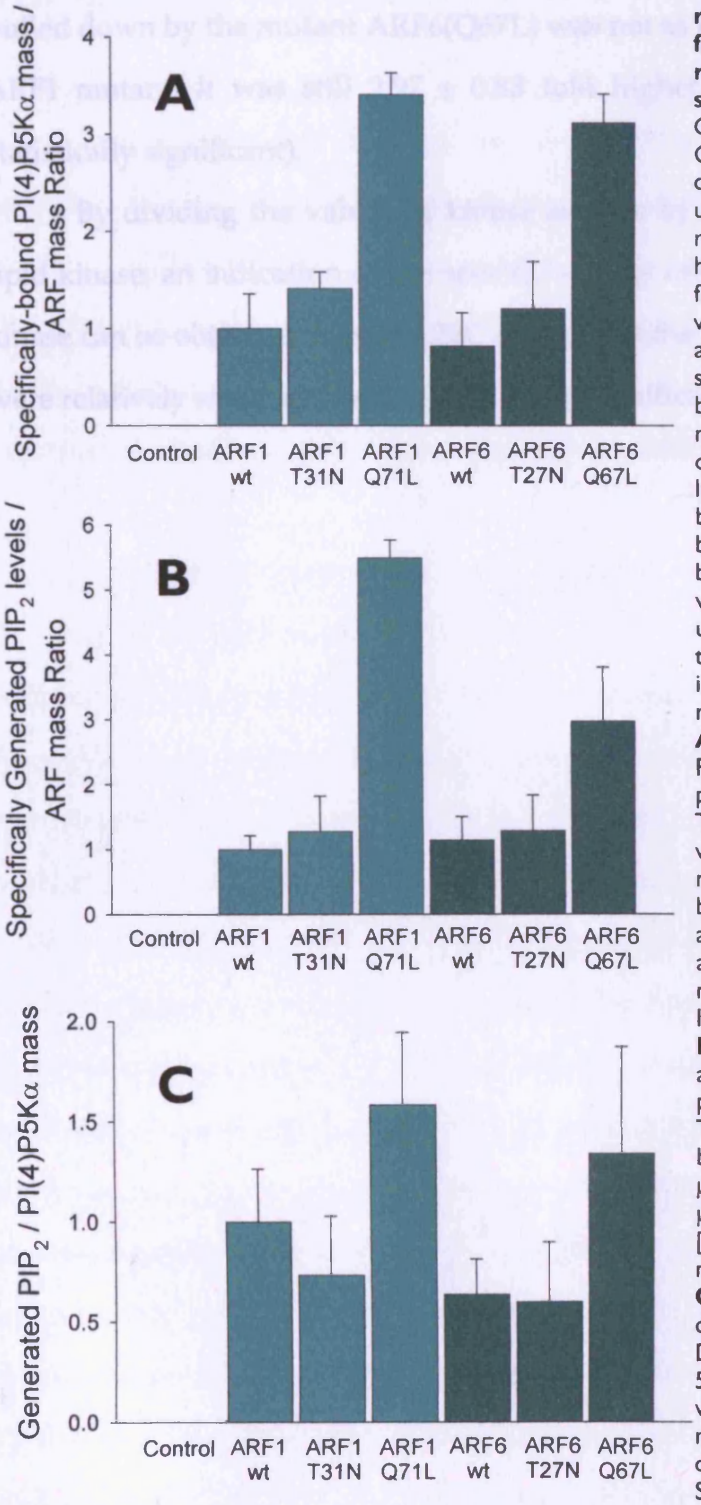


Figure 4.22 Pull down of recombinant PI(4)P5K Iα from Cos7 cell lysates using recombinant ARF proteins on sepharose beads.

Cos7 cells were transfected with GST-PI(4)P5K Iα or with different HA-ARF constructs using Polyfect transfection reagent, left to express for 48 hours, lysed, centrifuged and frozen in small aliquots. HA-ARFs were immunoprecipitated using anti-HA antibody-coated sepharose beads (previously blocked for 1 hour at 4°C with 5 mg/ml BSA), washed and put in contact with GST-PI(4)P5K Iα lysates for 1 hour at 4°C. The beads were then washed in lysis buffer and one last time in kinase buffer, and resuspended in a final volume of 100 µl. 10 µl were used to assay kinase activity and the remaining 90 µl were boiled in SDS-PAGE sample buffer and run on a 10% polyacrilamide gel.

A. Specifically bound amount of PI(4)P5K Iα per amount of ARF protein used in the pull down. The amount of ARF on the gel was quantified using monoclonal mouse anti-HA antibody, of beads/anti-HA antibody using anti-mouse secondary antibody and of PI(4)P5K Iα using monoclonal rabbit antiPI(4)P5K Iα antibody.

B. Specifically bound kinase activity per amount of ARF protein used in the pull down. The kinase assay was performed by addition to the beads of 80 µM PI(4)P (DMPC) vesicles, 50 µM MgATP and 5µCi/reaction of [γ-^{32}P]ATP, and was run for 20 minutes at room temperature.

C. Specific activity of the pulled down kinase. B/A values.

Data shown are mean ± S.E.M. of 5 separate experiments within which single measurements were made. The statistical significance of the data was evaluated using Student's *t* test.

5.49 ± 0.27 fold of wild type (P=0.0001/n=5). Strikingly, the activity of the kinase pulled down by the mutant ARF6(Q67L) was not as high as that of the equivalent ARF1 mutant. It was still 2.97 ± 0.83 fold higher than wild type ARF1 (not statistically significant).

By dividing the values of kinase activity by the amount of pulled down lipid kinase, an indication of the specific activity of the specifically pulled-down kinase can be obtained. Figure 4.22C shows that the variations in specific activity were relatively small and with no statistical significance.

4.3 DISCUSSION

This chapter has focused on the comparative activation of Type I phosphatidylinositol 4-phosphate 5-kinase enzymes by Class I and III ARF proteins, ARF1 and ARF6 respectively. To my knowledge, this is the first time that such a rigorous study of the differences in the activation of Type I PI(4)P5K by these two ARF isoforms has been carried out, by *in vitro* enzymatic assays, permeabilised-cell assays and coimmunoprecipitation experiments.

Honda *et al.* were the first to establish a direct activation of murine PI(4)P5K Iα by ARF *in vitro*. Previously, there had been reports showing that addition of ARF proteins increased the production of $PI(4,5)P_2$ in cell membranes (Fensome *et al.*, 1996; Martin *et al.*, 1996) and in the Golgi complex, by recruitment of an unidentified Type I PI(4)P5K (Godi *et al.*, 1999). Later on, another one of the three **isoforms** of the lipid kinase, PI(4)P5K Iγ, was found to also be activated by ARF6 in neurons (Krauss *et al.*, 2003; Aikawa and Martin, 2003). In order to see whether the activation by ARF proteins was common to the three known isoforms of Type I PI(4)P5K, the enzymes were mixed individually with recombinant ARF proteins. It was patently clear that all three isoforms of lipid kinase, α, β and γ, were activated by both ARF1 and ARF6, but that this was always dependent on the presence of the myristate group and of the non-hydrolysable guanosine triphosphate nucleotide. There was only one instance where the myristate group was not strictly necessary for the activation of the lipid kinase by ARF6, in the case of the recombinant murine β isoform expressed in *E. coli* (GTPγS and the myristoylation of ARF6 improved its activation though). This isoform seemed to be more sensitive to activation by ARF6 regardless of its

myristoylation state and even in the absence of GTPγS. It could be that the β isoform interacts more easily with a region of ARF6 that does not require rearrangement upon GTP loading (outside switch or inter-switch regions) but not with ARF1. This characteristic would differentiate the murine β (human α) isoform from the other two, perhaps revealing the possibility of specialised regulation by ARF6. Murine PI(4)P5K Iβ, which corresponds to human PI(4)P5K Iα, is the isoform that is thought to be located on the plasma membrane, where ARF6 is also thought to be concentrated.

There is only one reference in the literature where the presence of the **myristate** group is considered to be necessary for the activation of Type I phosphatidylinositol 4-phosphate 5-kinase. Honda *et al* presume this requirement when comparing the activation of Type I PI(4)P5K by their 10%-50% myristoylated recombinant ARF1 protein with the considerably stronger activation achieved with the isolated native protein. In the rest of the published studies of PI(4)P5K activation by ARF proteins, the use of partially myristoylated proteins is a widespread practice. Biochemical studies have established that, following activation, ARF-GTP exposes its myristoylated N-terminus and interacts with membranes via both the myristoyl group and hydrophobic and basic residues from the N-terminal α-helix. Myristoylation of ARF6 has been shown to be necessary for its interaction with membranes, as around only 5% of the nonmyristoylated protein can actually interact with lipid bilayers (D'Souza-Schorey and Stahl, 1995). No significant binding is detectable when ARF is not myristoylated or when anionic phospholipids (PG or PS, but not PIP_2) are absent from the vesicles (Antonny *et al.*, 1997). On the other hand, it has also been reported that the presence of the myristate group is only necessary for the membrane binding of the GDP-bound form of ARF1, whereas in the case of GTP-

ARF1, the acyl group is dispensable. Its presence would therefore be necessary for ARF1 to interact with its exchange factors on the membrane, which would catalyse the interchange of GDP for GTP and thus activate the small GTP binding protein (Haun *et al.*, 1993; Franco *et al.*, 1993, 1995, 1996). In the cell-free experiments performed throughout this investigation, there are no exchange factors to catalyse this reaction (its inclusion proved to be pointless under the conditions in use) or non-anionic lipids (PG or PS) included in the vesicles, therefore, no significant interaction between these vesicles and nonmyristoylated ARF proteins should have been expected. In accordance with this, the experiments clearly revealed that in the absence of the myristate group, ARF proteins were not capable of activating Type I PI(4)P5K under conditions equivalent to those where an activation occurs in the presence of the myristate group, for instance, at 1 μM ARF and 25°C-incubation conditions. It is reasonable to assume that this is a consequence of the failure of nonmyristoylated ARF to increase the tethering of its effector to the vesicles.

When the amount of recombinant nonmyristoylated ARF proteins was augmented to increase the probability of co-localising both proteins onto vesicles by mass action, no activation of PI(4)P5K was seen if the reaction was performed at 25°C. Not only was there not an improvement but, in the case of nonmyristoylated ARF1 and Δ17ARF1 there was a decrease in the production of $PI(4,5)P_2$ below the control levels which did not take place with ARF6, or at least not as markedly. Only when fully GDP- or GppNHp-loaded ARF proteins were used, exclusively at 37°C and at high concentrations (10 μM), was the activation of PI(4)P5K seen with myristoylated ARF proteins recapitulated. Conceivably, this is a consequence of a temperature- and mass-action-driven effect. There is a large molar excess of ARF over PI(4)P5K. Moreover, temperature has been shown to improve the binding of ARF molecules to vesicles (Haun *et al.*, 1993),

thus possibly increasing the strength and duration of the interaction of ARF-PI(4)P5K with the lipids and consequently allowing for a more prolonged generation of $PI(4,5)P_2$ even in the absence of the myristate group.

In comparative terms and under all the assayed conditions, ARF6 is always a better activator of Type I phosphatidylinositol 4-phosphate 5-kinase than ARF1. From experiments performed by collaborating colleagues we know that spontaneous GTP-loading by ARF6 is 2 to 3 times more efficient than ARF1 in the absence of ARF-GEFs (Ha, 2003). To rule out the possibility that the recurrent preference is a consequence of better nucleotide loading, fully GDP- or GTP-loaded nonmyristoylated ARFs were used. By using these proteins, the ambiguities due to differences in GTP-loading can be eliminated, although in the necessary absence of N-myristoylation the affinity of ARF (or any complexes it forms) for lipid vesicles is probably reduced. Hence, this system will primarily reflect the degree of ARF-PI(4)P5K binding (and catalytic activation if existent) rather than recruitment onto vesicles. This experiment showed that even then, ARF6 managed to stimulate PI(4)P5K more efficiently than ARF1 presumably due to a preferential binding to PI(4)P5K of ARF6 over ARF1. This preference is further supported by equivalent experiments using a different lipid kinase, p110γ, where there is no apparent preference and which will be discussed later on in this thesis.

Only twice in the literature have these two isoforms of ARF been compared in their potency to activate PI(4)P5K Iα. Honda *et al.* compared the activation *in vitro* of bacterially-expressed recombinant PI(4P)5K Iα by ARF1, 5 and 6 expressed in Cos7 cells, which would therefore be expected to be 100% myristoylated. ARF1 and 6 provoked a similar five fold activation of the lipid kinase whereas ARF5 increased activity seven fold. From the experiments in this

chapter it became clear that there are certain differences in behaviour depending on the origin of the recombinant Type I PI(4)P5K. The enzyme expressed in bacterial systems is much more responsive to the influence of both ARF1 and 6, showing little difference in activation by both isoforms. Nevertheless, when the lipid kinase is expressed in a mammalian system (and therefore subjected to all the post-translational changes carried out in mammalian cells) the activation by both isoforms of ARF is importantly diminished, especially by ARF1. Therefore, especial care should be taken when drawing conclusions based on bacterial recombinant PI(4)P5K, and further characterisation of the effects should be established by comparison with mammalian recombinant enzymes.

The other example in the literature of differential activation of Type I PI(4)P5K by ARF proteins was carried out by Skippen *et al.* (2002), who also compared the two ARF isoforms in HL60 cells and concluded that they were equally effective in restoring $PI(4,5)P_2$ synthesis. However, their ARF proteins were partially myristoylated and they did not state to what percentage or whether the fractional derivatisation was equivalent or different. Consequently, given the triple impact of myristoylation on nucleotide exchange rates, membrane affinity and effector activation (discussed here) then the comparison cannot be considered technically valid. Moreover, they confirmed that most of the $PI(4,5)P_2$ produced in HL60 cells by readdition of ARF1 was generated at the plasma membrane since it was brefeldin A resistant, that is, under the control of the low molecular weight family of ARF-guanine nucleotide exchange factors (GEFs) that reside in this compartment (Jackson and Casanova, 2000). ARF6 is thought to be the only isoform of ARF present at the plasma membrane, whereas ARF1 is mostly Golgi-localised if not cytosolic (Peters *et al.*, 1995). Therefore, it is possible that the activation of Type I PI(4)P5K by ARF1 may not be its normal function, although due to the large amount added to the permeabilised cells, it

compensates for the absent ARF6 at the plasma membrane. When similar experiments were performed using permeabilised HL60 cells and 100% myristoylated recombinant ARF1 and ARF6, there was also an increase in $PI(4,5)P_2$ production by both isoforms, but again this was stronger in the case of ARF6.

Finally, a direct interaction between ARF and PI(4)P5K Iα was demonstrated by co-precipitation experiments. Separate expression of the recombinant proteins and their subsequent combination allowed for the recovery of phosphatidylinositol 4-phosphate 5-kinase Iα by interaction with the HA-tagged ARF proteins. The level of interaction was similar with the two ARF isoforms under study, ARF1 and ARF6. Both the wild type and the dominant-negative mutants pulled down similar amounts of kinase, while the constitutively active mutants were much more efficient in doing so. This shows that PI(4)P5K Iα is capable of binding ARF in any of its activation states, but the interaction is more efficient when it is GTP-bound. It is possible that the interaction of PI(4)P5K Iα with ARF happens in part with residues outside the switch and inter-switch region, but the rearrangements of ARF switch 1 and 2 induced upon nucleotide exchange strengthen the interface contact. Solution of the GDP- and GTP-bound structures of ARF1 and ARF6 from single crystal X-ray diffraction data proved that there was little structural difference between the active form of the two proteins, but that the switch regions of the inactive GDP-bound conformation is the discriminatory factor between the two isoforms (Pasqualato *et al.*, 2001). This could suggest that GTP-bound ARFs may establish specific interactions outside the switch regions and/or be recognised differently in their cellular context rather than as isolated proteins. In this pull-down experiment there are no natural membranes and therefore the cellular context

has been distorted, and so the interaction we can see is the pure structural one rather than the one that may happen within cells. Hence the interaction here is devoid of the influence of the cellular localization and of the multiple effectors that control the functioning of these proteins. In the case of the interaction of ARF with PLD, it has been established that it occurs with the N-terminal helix and the following loop, the α2 helix and part of the β2 strand (Jones *et al.*, 1999; Sung *et al.*, 1999). These residues become exposed after GTP-binding, and they are similar contact sites to those involved in the interaction with exchange factors (Goldberg, 1998). These data must be directly compared with the interaction of PI(4)P5K with other small G proteins. Both Rac1 and RhoA, (but not Cdc42), have been shown to interact with the lipid kinase in a GTP-independent manner, through binding with the C-terminal region of the GTPases (Tolias *et al.*, 1995; Ren *et al.*, 1996; van Hennik *et al.*, 2003; Weernink *et al.*, 2004). Consequently their function is no other than recruitment of the lipid kinase onto membranes, and not catalytic activation of the enzyme. It seems conceivable that this is the case with ARF as well: binding of the two proteins is nucleotide-independent (see the effect of fully nucleotide-loaded ARF proteins), but apparent activation is GTP-dependent due to the necessity to tether PI(4)P5K to the membrane where its substrate lies.

The novel aspect of this experiment is the simultaneous assay of kinase activity pulled down with the ARF isoforms. The constitutively active isoforms pulled down a higher PI(4)P phosphorylating activity. However, it was intriguing to see that the amount of $PI(4,5)P_2$ produced by the ARF1(Q71L)-associated kinase was more than that associated with the ARF6 mutant. In accordance with my *in vitro* and permeabilised-cell assays, it would have been expected to be the other way around. Nevertheless, there is a major difference in the way the interaction is presented in the different systems. In the *in vitro*

activation assays, ARF was always free in solution and PI(4)P5K was either in solution or bound to beads, which makes little difference on the activation by ARF; if anything, there was a slight improvement when PI(4)P5K was free in solution, probably because the freely diffusible state favours the interaction between the two proteins by better co-localisation on the lipidic surface. In this case, it is ARF that is bound to beads and cannot diffuse freely in the reaction solution. The stoichiometry of the interactions of these two proteins is unknown. Blocking the free movement of ARF restricts the way the interaction takes place. Type I PI(4)P5Ks are known to associate in homo and heterodimers and most probably, in oligomeric protein complexes. In the case of PI(4)P5K Iβ, gel filtration analysis revealed that the kinase is present in high molecular weight protein complexes (Galiano *et al.*, 2002). On the other hand, it has been suggested that ARF1 may also form dimers during its biological function due to the occurrence of a dimer in one crystal form (Greasley *et al.*, 1995). These data taken together add multiple possibilities to the way these two proteins could interact, therefore making it a potentially complex situation when it comes to comparing activation and interaction using different experimental methods, for these might limit the possibilities found in living cells. This is probably the reason why there is a clear contradiction between the only two examples of PI(4)P5K and ARF interaction in the literature. Krauss *et al.* (2003) used an affinity purification approach to try to demonstrate an interaction between ARF and PI(4)P5K Iγ. They managed to pull down the γ isoform from brain extracts by interaction with ARF6(Q67L) exclusively, but they saw no interaction with the GTP-binding suppressed mutant ARF6(T27N). They also tried to use the ARF1(Q71L) mutant and they concluded that there was no interaction with PI(4)P5K Iγ, even though in the blot presented in the article there was a band corresponding to the pulled down kinase. The contradiction was exposed a month later when Aikawa and

Martin published data on the coimmunoprecipitation of ARF6 with PI(4)P5K Iγ after coexpressing the two proteins in PC12 cells. They found that the mutants ARF6(Q76L) and ARF6(N122I) (another GTP-binding deficient mutant) exhibited similar associations with PI(4)P5K Iγ, which where enhanced by Ca^{2+} influx. They concluded that the interaction between ARF6 and PI(4)P5K is regulated by a Ca^{2+}-dependent mechanism, involving the activation of PKC and the phosphatase PP1, with the consequent dephosphorylation of PI(4)P5K as previously established by Park *et al.* (2001). All in all, the interaction between ARF6 and PI(4)P5K appeared to be governed principally by the phosphorylation state of PI(4)P5K rather than the guanine nucleotide-bound state of ARF6. These results are in direct conflict with those of Krauss *et al.*, despite the fact that both use the same PI(4)P5K and ARF isoforms. The different methods used to look for interaction may be the cause for the discrepancy in the results.

In addition, since the interaction between the two proteins has been obtained in a system where the amount of recombinant proteins is in excess due to overexpression, the result could be distorting the real situation that takes place in physiological conditions, forcing a higher degree of interaction. Furthermore, the use of these mutants blocks the normal cycle of GTP binding, hydrolysis, and release. Therefore, although the GTPase-deficient mutants are in the active conformation, they do not fully imitate all the actions of the GTPase (Santy, 2002). Finally, in this case recombinant ARF proteins had been expressed in mammalian cells whereas in previous instances they were of bacterial origin. All these factors could explain why in this case ARF6 is not more efficient in activating PI(4)P5K, as opposed to the previous experiment. In conclusion, this pull-down experiment should be considered as a demonstration that a direct interaction between the two proteins takes place, but not as a reference of comparative binding or activation by different isoforms of ARF.

In the same line but in terms of specific activation, there are no statistically significant differences between the different ARF mutants. Therefore, it could be concluded that ARF proteins are responsible for binding and recruiting PI(4)P5K onto membranes, but not of catalytically activating it, as it is also the case with GTPases of the Rho family.

Considering them together, the results presented in this chapter clearly highlight myristoylation as a critical feature in the activation of Type I PI(4)P5K by ARF proteins, without which ARF is not capable of activating the lipid kinase and at high concentrations, it can cause the opposite effect on the generation of $PI(4,5)P_2$. Hence optimal data in biochemical assays using recombinant ARF proteins are highly dependent on the generation of fully-myristoylated ARF proteins and failure to achieve this may devalue the relevance of any data obtained. Also, reports based on the use of recombinant Type I PI(4)P5K expressed in bacteria should be carefully evaluated because its behaviour is not exactly that of the same enzyme expressed in mammalian cells, conceivably due to the absence of post-translational modifications. Finally, the comparison of the effects of ARF1 and ARF6 on Type I PI(4)P5K strongly points to a role for ARF6 and not ARF1 as the factor that directly regulates PI(4)P5K Iα in permeabilised cells and cell-free model systems and therefore most probably also *in vivo*. This statement is supported by several articles where ARF6 and PI(4)P5K or regions with high $PI(4,5)P_2$ concentration have been shown to co-localise in living cells (Brown *et al.*, 2001, Krauss *et al.*, 2003, Aikawa and Martin, 2003), while ARF1 and PI(4)P5K do not (Honda *et al.*, 1999).

Chapter Five

Regulation of Type I PI(4)P5Kα by Diacylglycerols

5.1 INTRODUCTION

Many stimuli such as hormones or growth factors provoke a transient increase in levels of cellular diacylglycerol through hydrolysis of $PI(4,5)P_2$ by phospholipase C. Many of these stimuli also promote the activation of phospholipase D with the consequent generation of phosphatidic acid through hydrolysis of phosphatidylcholine, which can be in turn converted into diacylglycerol by phosphatidate phosphohydrolases. DAG is a renowned second messenger, being the activation of PKC enzymes, specifically members of the conventional and novel subgroups, one of its best characterised effects. However, it may also have other cellular targets such as α- and β-chimaerins, guanine nucleotide-exchange factors for Ras and Rap, nonselective cation channels, Munc-13, DGKα, β and γ, and PKD isozymes (van Blitterswijk and Houssa, 2000; Brose and Rosenmund, 2002; Yang and Kazanietz, 2003).

Because sequence analysis predicted a potential PKC phosphorylation site within phosphatidylinositol 4-phosphate 5-kinase enzymes, I made an attempt to characterise the effects of PKCs on the activity of the lipid kinase.

During the course of these experiments, I made the novel discovery of a possible direct activation of phosphatidylinositol 4-phosphate 5-kinase by DAG that stimulates the production of $PI(4,5)P_2$.

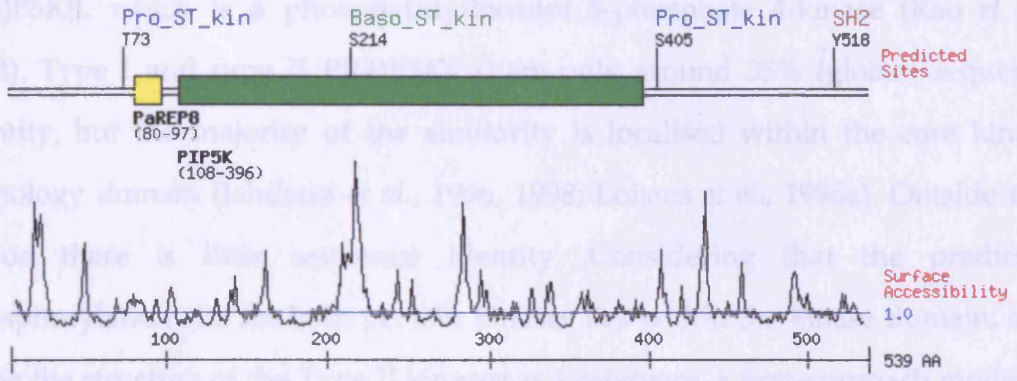
Finding a DAG-dependent regulation of PI(4)P5Ks in addition to that mediated by PKCs would even more firmly place these lipid kinases directly downstream of the activation of PLC enzymes. Because PLCs cause $PI(4,5)P_2$ hydrolysis upon reception of a stimulus, then this would create a very effective mechanism of replenishing the vital polyphosphoinositide.

5.2 RESULTS

Existence Of A Potential Phosphorylation Site For PKCα and PKA Within The Sequence Of PI(4)P5K Iα

One of the highly specific control methods utilised inside cells to transduce signals, activate and inactivate proteins and produce signalling cascades is protein phosphorylation, either on tyrosine or serine/threonine residues. There are more than 500 protein kinases in mammalian genomes and all of them usually alter in some way the localisation, activity and function of other proteins. The activation of these kinases is often the consequence of some extracellular signal.

In an attempt to determine whether Type I PI(4)P5Ks could be regulated by phosphorylation by protein kinases, an analysis of the amino acid sequence of murine PI(4)P5K Iα was carried out using a range of bioinformatic resources. For example, Scansite (Obenauer *et al.*, 2003), predicted some statistically probable phosphorylation sites. Table 5.1 shows the predicted sites for a group of serine/threonine kinases, including PKC and PKA. There are other phosphorylation sites predicted such as those for Cdk5 kinase and Cdc2 kinase of the proline-dependent serine/threonine kinases, but they are not tabulated as they are not relevant to this study. As observed, there are good potential consensus phosphorylation sites for PKCα and PKA both at serine 214. The percentile value given for the sequence regarding potential phosphorylation by PKCα is lower than that for PKA, but the internal score obtained for PKA phosphorylation is slightly lower than that for PKC. So both protein kinases are predicted to be reasonable phosphorylating agents for the lipid kinase according



High Stringency					
Enzyme	Residue	Score	Percentile	S.A.	Sequence
PKC α/β/γ	S214	0.3687	0.089%	4.122	STYKRRA**S**RKEREKP

Medium Stringency					
Enzyme	Residue	Score	Percentile	S.A.	Sequence
PKA	S214	0.3650	0.266%	4.122	STYKRRA**S**RKEREKP
PKC α/β/γ	S214	0.3687	0.089%	4.122	STYKRRA**S**RKEREKP
PKCε	S468	0.4252	0.502%	0.848	DLVPSTP**S**LFEAASL
PKCδ	S26	0.4349	0.521%	0.425	KTYKKTA**S**SAIKGAI
PKCε	T224	0.4408	0.716%	2.470	EREKPNP**T**FKDLDFL
PKCζ	S393	0.4934	0.355%	0.639	RFLKFMN**S**RVFKKIQ

Table 5.1 Prediction of phosphorylation sites for basophilic serine/threonine kinases on the sequence of murine PI(4)P5K Iα using *Scansite*.
The *Score* value starts at 0.000 if the sequence optimally matches the given motif, and it increases for sequences as they diverge from the optimal match. The *Percentile* value is representative of how much better is the selected sequence as a target for the specific kinase in comparison to the rest of existent similar consensus sequences in the database (i.e. a 0.089% value means that this sequence is better than 99.911% of sequences in the SwissProt database as a theoretical phosphorylation site). A high stringency search will only collect hits with percentile values below a threshold of 0.2%. The *S.A.* value is representative of the surface accessibility of the amino acid residue; the higher the value over 1, the more exposed the residue. This value is obtained from the amino acid sequence and not with reference to any known structures.
The figure on top of the tables represents the PFAM-predicted domains and predicted phosphorylation sites of murine PI(4)P5K Iα using the high stringency search and below is the surface accessibility chart.

to this particular bioinformatic analysis.

The structure of Type I PI(4)P5Ks has not been elucidated to date. The only known structure of a significantly similar kinase is that of Type II

PI(4)P5Kβ, which is a phosphatidylinositol 5-phosphate 4-kinase (Rao *et al.*, 1998). Type I and type II PI(4)P5Ks share only around 35% (global) sequence identity, but the majority of the similarity is localised within the core kinase homology domain (Ishihara *et al.*, 1996, 1998; Loijens *et al.*, 1996a). Outside this region there is little sequence identity. Considering that the predicted phosphorylation site for both protein kinases lies within the kinase domain, and using the structure of the Type II kinases as a reference, a first-approach model of the structure of Type I enzymes was generated using SwissModel. In this model, the position of serine 214 could be predicted to be located in the external surface of the protein close to the catalytic site of the lipid kinase as seen in Figure 5.1.

PKCα Inhibits Type I PI(4)P5Ka Expressed in *E. coli*

Given a good match to a consensus phosphorylation site and likely surface exposure assessed by both model building and sequence analysis, the following experiment was developed to assay the possibility of an effect of recombinant PKCα on recombinant PI(4)P5K Iα expressed in bacteria. In order for PKCα to function correctly it requires the addition of certain effectors, namely phosphatidylserine, diacylglycerol and Ca^{2+}. These effectors can be added with the protein kinase into the reaction mixture that includes the target substrate protein. However, in this particular assay a problem arises because at least one of these effectors, PS, is a known activator of Type I PI(4)P5K (Cochet and Chambaz, 1986). This means that the relative effect of PKC on the activity of the lipid kinase, quantified by measuring changes in the generation of $PI(4,5)P_2$, might be masked by the effect of PS if both protein phosphorylation and lipid phosphorylation reactions were performed simultaneously or sequentially in the same reaction mixtures. For this reason, it was imperative to develop a method that would allow for the separation of the two steps of the reaction. Initially,

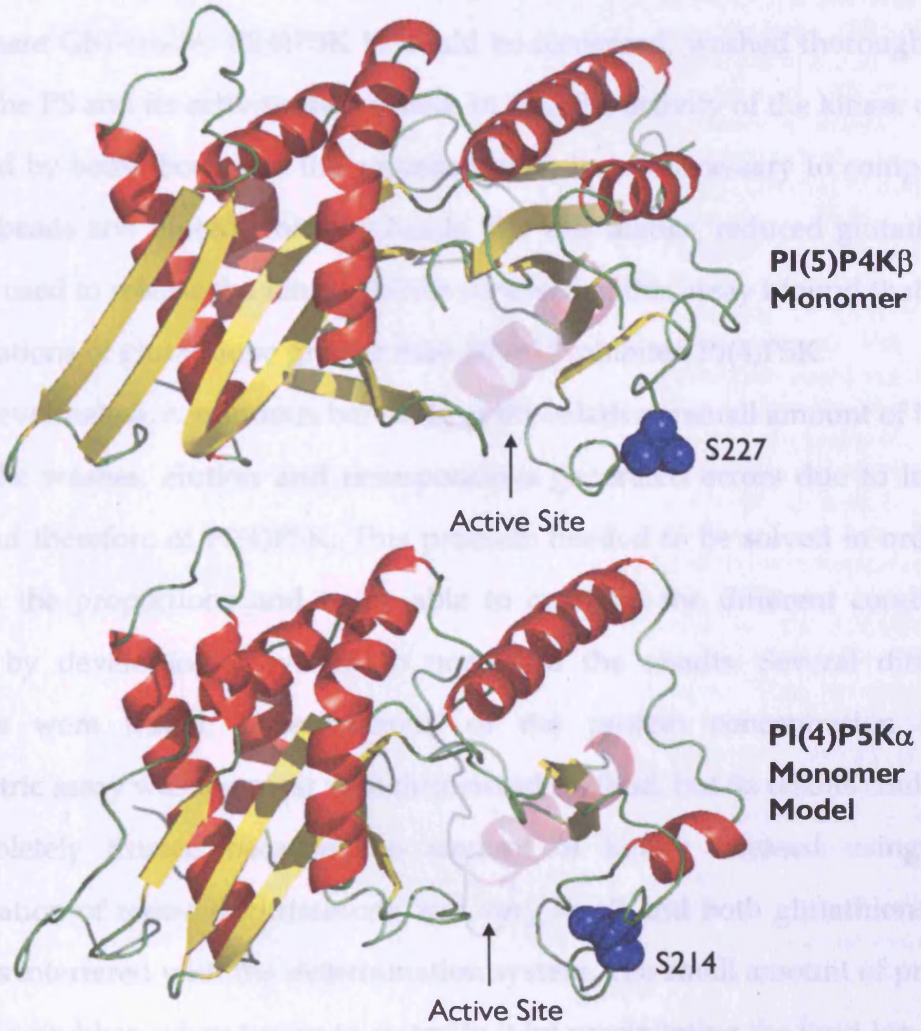


Figure 5.1 **Crystal structures of a monomer of PI(5)P4Kβ and a model of PI(4)P5Kα.**
Same sagittal projection of a PI(5)P4Kβ monomer (human PI(5)P4Kβ; PDB 1BO1) and a model of a PI(4)P5Kα monomer generated using SwissModel. The location of the potential PKC/PKA phosphorylation Serine is indicated in blue spacefill in both monomers. The location of the interacting membrane would be beneath the structure.

PKC, its effectors and PI(4)P5K were mixed together to allow PKC to phosphorylate the lipid kinase. Subsequently, PI(4)P5K Iα had to be extracted from the mixture, the effectors washed off and the lipid kinase mixed with its substrates, PI(4)P and MgATP, to allow for quantification of its phosphorylation

capacity. By adding glutathione coated agarose beads to the first mixture, the recombinant GST-fusion PI(4)P5K Iα could be recovered, washed thoroughly to remove the PS and its activity then tested. In case the activity of the kinase could be altered by being bound to the agarose beads, it was necessary to compare it both on beads and eluted from the beads. For this matter, reduced glutathione could be used to release the kinase. While developing this assay I found that final concentrations of glutathione greater than 10 mM inhibited PI(4)P5K.

Nevertheless, continuous handling of the relatively small amount of beads during the washes, elution and resuspensions generated errors due to loss of beads and therefore of PI(4)P5K. This problem needed to be solved in order to maintain the proportions and to be able to compare the different conditions assayed by developing a system to normalise the results. Several different strategies were tested. Determination of the protein concentration by a colorimetric assay was the most straightforward method, but its results could not be completely trusted because the amount of kinase released using low concentration of reduced glutathione was very small and both glutathione and the lipids interfered with the determination system. The small amount of protein was also a problem when trying to quantify it by precipitating the lipid kinase to separate it from the interfering substances, using acetone or trichloroacetic acid precipitation methods. These approaches also yielded negative results. Other methods such as ^{3}H-NSP-labelling (N-succinimidyl-[2,3-^{3}H]-propionate) of the protein were tested but without success.

Finally, I used the glutathione S-transferase activity associated with the GST-fusion protein as a direct indicator of protein concentration. This was done by measuring the rate of formation of a yellow product at 340 nm after addition of reduced glutathione (1 mM final), 1-chloro-2,4-Dinitrobenzene (CDNB, 1 mM) and potassium phosphate buffer pH 6.5 (10 mM final) at room temperature. The

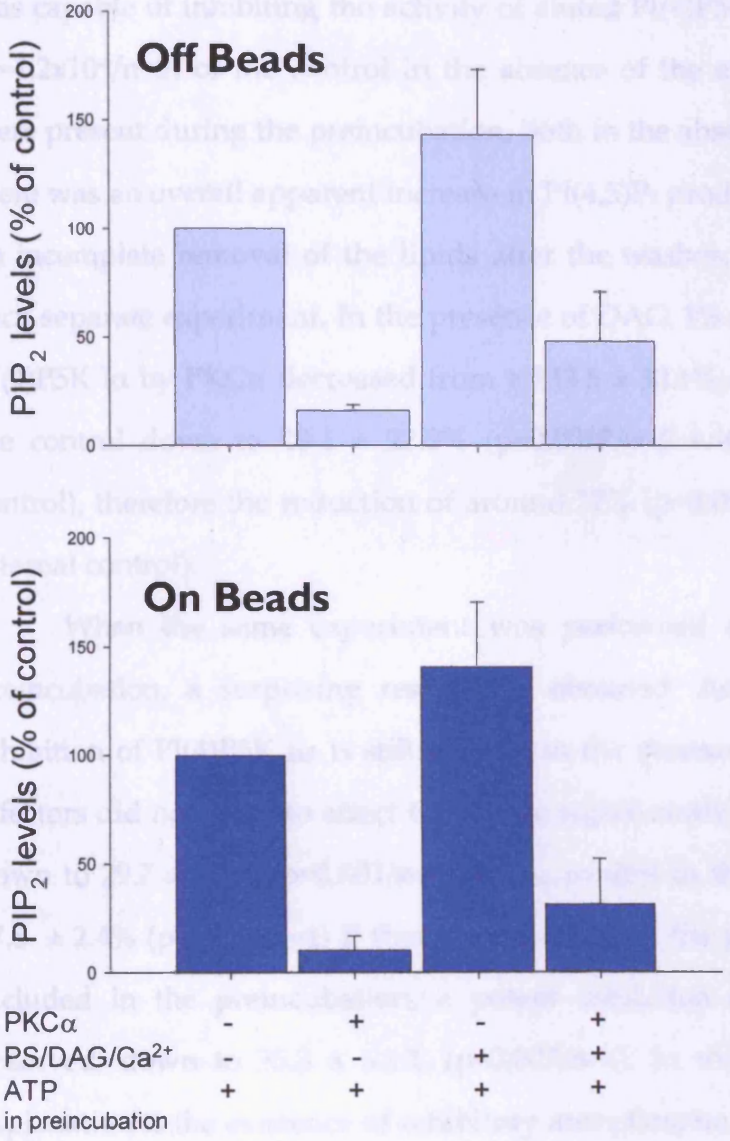


Figure 5.2 ***In vitro* effect of PKCα and its effectors on recombinant PI(4)P5K Iα expressed in *E. coli*.**
A sample of GST-PI(4)P5K Iα expressed in bacteria was taken and incubated for 1 hour at 37°C with 75 μM MgATP and with or without PKCα and/or PS/DAG/Ca^{2+}. The beads were then recovered, washed thoroughly in microfilters and 200 μl of 10 mM reduced glutathione added to elute part of the kinase for 15 minutes at room temperature. The supernatant was filtered through and 4x30 μl used in a typical kinase assay. Another 65 μl were used to normalise the amount of protein by performing a GST assay with 6.5 μl of CDNB, 71.5 μl of buffer and 572 μl of distilled water. The remaining beads were resuspended in kinase buffer and assayed for kinase activity as well for 20 minutes at room temperature as described in the Materials and Methods section.
Data shown are mean ± S.E.M. of 5 separate experiments within which measurements were made in quadruplicate. The statistical significance of the data was evaluated using Student's *t* test.

rate of colour change was directly proportional to the amount of GST fusion protein present in the solution and this allowed for a direct estimation of the amount of PI(4)P5K.

Figure 5.2 shows the effect of commercial recombinant PKCα on recombinant GST-tagged PI(4)P5K Iα expressed in *E. coli*. As expected from the results on the previous chapter, assaying the kinase activity on beads or free in solution did not make a significant difference in the results. Recombinant PKCα

was capable of inhibiting the activity of eluted PI(4)P5K Iα down to 16.2 ± 2.6% ($p=1.2x10^{-6}/n=5$) of the control in the absence of the effectors. If these effectors were present during the preincubation, both in the absence or presence of PKCα, there was an overall apparent increase in $PI(4,5)P_2$ production presumably due to an incomplete removal of the lipids after the washes, which tended to vary in each separate experiment. In the presence of DAG, PS and Ca^{2+}, the inhibition of PI(4)P5K Iα by PKCα decreased from a 143.5 ± 30.1% of activity with respect to the control down to 48.1 ± 22.8% ($p=0.0017/n=5$ with respect to the general control), therefore the reduction of around 77% ($p=0.049/n=5$ with respect to the internal control).

When the same experiment was performed excluding ATP from the preincubation, a surprising result was obtained. As seen in Figure 5.3, the inhibition of PI(4)P5K Iα is still present in the absence of ATP. In this case the effectors did not seem to affect the kinase significantly, and $PI(4,5)P_2$ levels were down to 29.7 ± 4.7% ($p=0.001/n=4$) of the control in their absence and down to 27.8 ± 2.4% ($p=0.001/n=4$) if they were present in the preincubation. If ATP was included in the preincubation, a potent inhibition of PI(4)P5K Iα was also observed, down to 35.3 ± 6.1% ($p=0.003/n=4$). In this case, the only possible explanation is the existence of inhibitory autophosphorylation. Type I PI(4)P5Ks are known to be able to autophosphorylate in serine/threonine residues attenuating their activity, effect that is markedly enhanced by the addition of PI (Itoh *et al.*, 2000). It is possible that the 1 hour preincubation in the presence of ATP is sufficient for the lipid kinase to increase its levels of autophosphorylation and consequently diminish its activity.

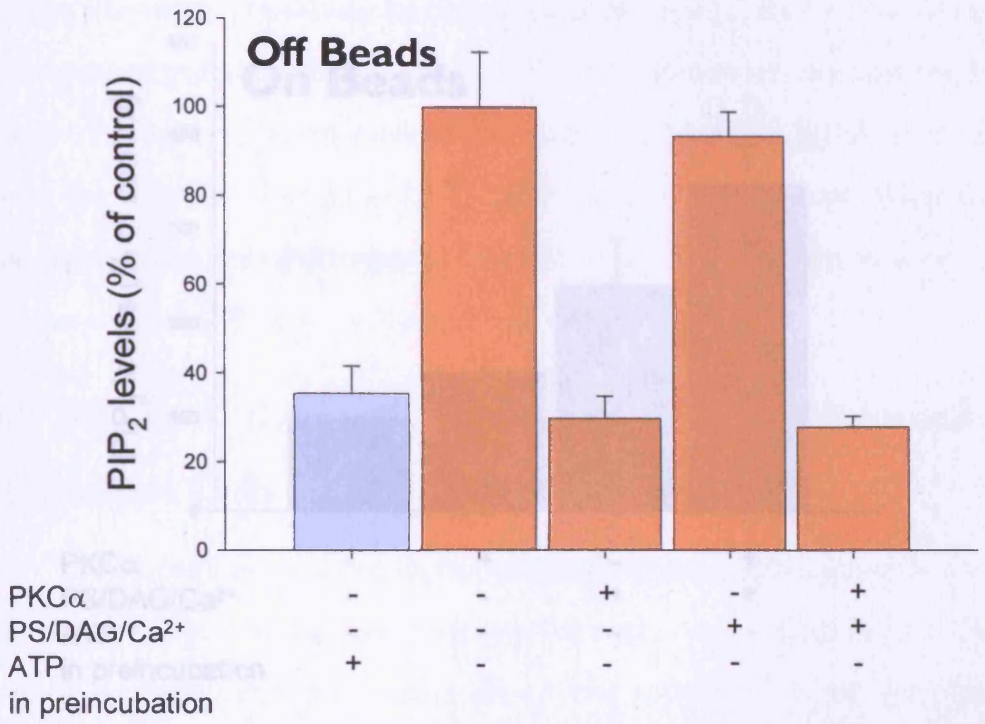


Figure 5.3 ***In vitro* effect of PKCα and its effectors on recombinant PI(4)P5K Iα expressed in *E. coli*.**

Recombinant Type I PI(4)P5K was expressed in *E. coli* as GST-tagged fusion protein and recovered on glutathione-sepharose beads. A sample of the kinase was taken and incubated for 1 hour at 37°C with or without 75 μM MgATP and/ or PKCα and/or PS/DAG/Ca^{2+}. The beads were then recovered, washed thoroughly in microfilters and 200 μl of 10 mM reduced glutathione added to elute part of the kinase for 15 minutes at room temperature. The supernatant was filtered through and 4x30 μl used in a typical kinase assay for 20 minutes at room temperature as described in the Materials and Methods section. Another 65 μl were used to normalise the amount of protein by performing a GST assay with 6.5 μl of CDNB, 71.5 μl of buffer and 572 μl of distilled water.

Data shown are mean ± S.E.M. of quadruplicate samples within a single experiment. The statistical significance of the data was evaluated using Student's *t* test.

PKCα Has No Effect On PI(4)P5K Iα Expressed In Mammalian Cells

When the same experiments were performed using HA-tagged PI(4)P5K Iα expressed in Cos7 cells no statistically significant differences were observed, as illustrated on Figure 5.4. This figure represents an experiment where the beads were not washed after the preincubation (to minimise handling-artefacts), which

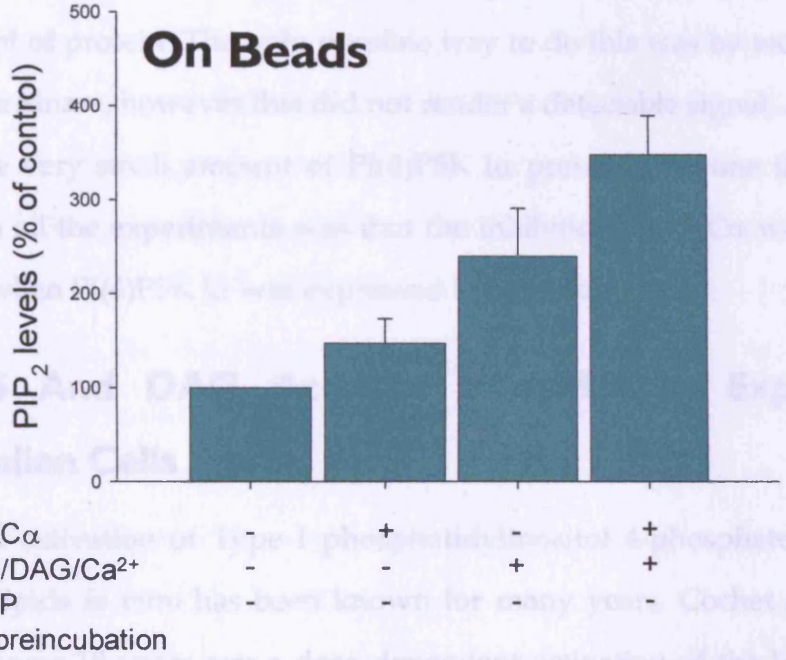


Figure 5.4 ***In vitro* effect of PKCα and its effectors on recombinant PI(4)P5K Iα expressed in Cos7 cells.**
Recombinant Type I PI(4)P5K was expressed in *Cos7* cells as HA-tagged fusion protein and recovered on anti-HA antibody-coated agarose beads. A sample of the kinase was taken and incubated for 1 hour at 37°C with or without PKCα and/or PS/DAG/Ca^{2+}. The beads were then split into 3x30 µl samples and the kinase activity was assayed for 20 minutes at room temperature as described in the Materials and Methods section.
Data shown are mean ± S.E.M. of 4 experiments within which measurements were made in triplicate. The statistical significance of the data was evaluated using Student's *t* test.

explains why in the presence of the effectors there was such an increase in the activity of PI(4)P5K. There was a statistically significant difference between the control and those conditions were the effectors were included, but the increase was due to their presence. There was no statistically significant difference between those conditions where PKCα was present or absent together with the effectors. The same experiment was performed introducing washes after the preincubation and including ATP, but again, variations in the data between experiments did not allow for an unambiguous result. However, these variations

were due to the impossibility of normalising the final data by quantification of the amount of protein. The only possible way to do this was by western blotting against the kinase, however this did not render a detectable signal, most certainly due to the very small amount of PI(4)P5K Iα present. The one thing that was clear from all the experiments was that the inhibition by PKCα was not present as it was when PI(4)P5K Iα was expressed in bacterial cells.

PA, PS And DAG Activate PI(4)P5K Iα Expressed In Mammalian Cells

The activation of Type I phosphatidylinositol 4-phosphate 5-kinases by different lipids *in vitro* has been known for many years. Cochet and Chambaz reported some 18 years ago a dose dependent activation of the lipid kinase by phosphatidylserine. 6 years later, Moritz *et al.* showed that phosphatidic acid was also a strong activator of the phosphoinositide kinase. These two lipids have been considered to be its main activators *in vitro* for a long time, but a direct proof of such activation *in vivo* has always been surrounded by certain degree of scepticism due to the intricate connections of the pathways that control the activity of Type I PI(4)P5K and the production of PA.

In the following experiment, the activation by these and other lipids was tested *in vitro* in an attempt to confirm the literature, as well as determining the exact origin of the stimulation of phosphoinositide kinase activity observed in the PKCα experiments. Figure 5.5 exemplifies the effect caused by the inclusion of different lipids in PI(4)P-containing vesicles, in the presence and absence of dimyristoyl-phosphatidylcholine as a carrier lipid and of two detergents with different chemical properties: the nonionic detergent Triton X-100 and the anionic detergent, sodium deoxycholate. These two detergents are both

nondenaturing agents. PI(4)P was always included in the mixtures at 80 μM concentration, and the different lipids under study were included in the vesicles at an equimolar concentration, i.e. 1:1 (PI(4)P/PA for example). PC was included at 500 μM, rendering an approximate molar ratio of, for instance, 6:1:1 (PC/PI(4)P/PA). It must be noted that control PI(4)P vesicles generated using the different detergents or PC varied the phosphorylation capacity of the lipid kinase, therefore the results were plotted relative to each internal control to account for such variations, and a representation of such values was plotted on the side for comparison (see inset in Figure 5.5). For instance, the sole inclusion of PC in the PI(4)P vesicles caused a 38 ± 10.7% ($p=0.0004/n=5$) decrease in the level of phosphorylation by PI(4)P5K Iα.

Both PA and PS were confirmed as activators of the lipid kinase, but only under some of the conditions examined. In those cases where the vesicles were prepared using detergents, no significant activation was achieved by either one of the two lipids. In fact, whenever deoxycholate was used to prepare the vesicles in combination with PC, an inhibition of PI(4)P5K Iα was observed below internal control levels, down to 41.1 ± 9.77% in the case of PA, 37.56 ± 10.41% with PS and 74.42 ± 5.54% with DAG ($p\leq0.0003,n=5$). The effect under all other conditions where detergents were present was not statistically significant.

PA activated PI(4)P5K Iα expressed in mammalian cells both on PI(4)P/PA vesicles, 290.97 ± 39.74% of control ($p=0.001/n=5$), and on PC/PI(4)P/PA vesicles, 181.93 ± 31.53 of internal control ($p=0.02/n=5$). This activation was also present in the case of PI(4)P/PS vesicles, up to 437.15 ± 38.7 ($p=2.35x10^{-5}/n=5$). However, when PC was included in the vesicles the PS effect disappeared completely.

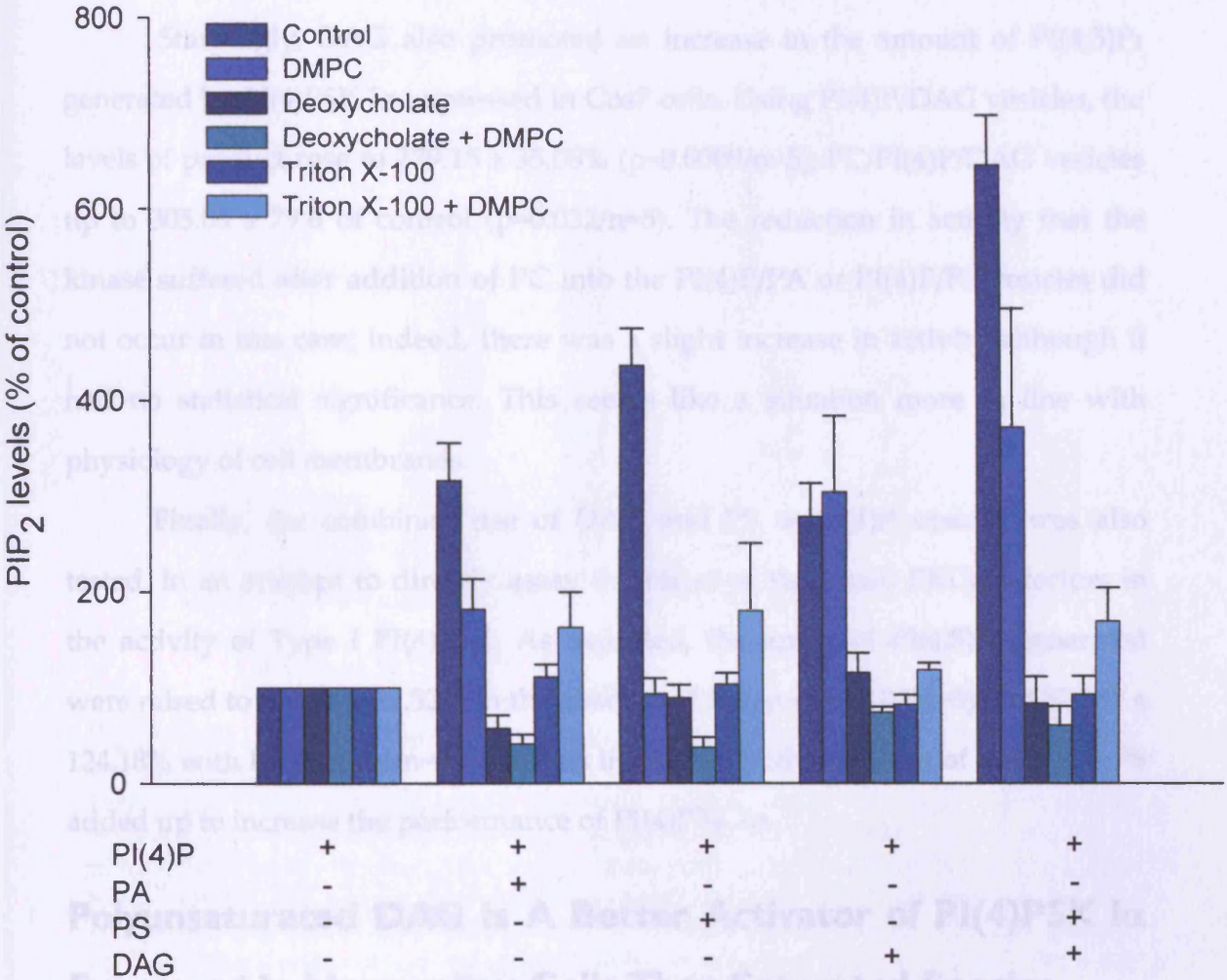
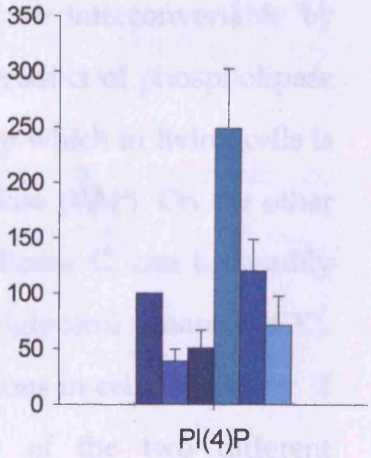


Figure 5.5 ***In vitro*** **activation of recombinant PI(4)P5K Iα expressed in Cos7 cells by different lipid mixtures.**
Recombinant PI(4)P5K Iα was expressed in Cos7 cells as HA-tagged fusion protein, recovered on anti-HA antibody-coated agarose beads, washed and stored in frozen aliquots. A sample of the kinase was taken and diluted in kinase buffer to obtain detectable phosphorylation levels. Different lipid vesicles were tested, composed of 80 μM PI(4)P and PA, DAG and/or PS, in combination with 500 μM DMPC as carrier lipid, and detergents such as Triton X-100 (0.1%) or deoxycholate (1mM). After 20 minutes at 25°C, the phosphorylation reaction was stopped by the addition of 80 μl of 10% HCl. PIP_2 levels were measured as described in the Materials and Methods section.
Data shown are mean ± S.E.M. of 5 separate experiments within which measurements were made in triplicate. For comparison, data are shown relative to the appropriate internal control since the absolute activity varied with substrate type (see insert). The statistical significance of the data was evaluated using Student's *t* test,



Startlingly, DAG also promoted an increase in the amount of $PI(4,5)P_2$ generated by PI(4)P5K Iα expressed in Cos7 cells. Using PI(4)P/DAG vesicles, the levels of product rose to 279.15 ± 35.08% (p=0.0009/n=5); PC/PI(4)P/DAG vesicles up to 305.05 ± 79.6 of control (p=0.032/n=5). The reduction in activity that the kinase suffered after addition of PC into the PI(4)P/PA or PI(4)P/PS vesicles did not occur in this case; indeed, there was a slight increase in activity although it had no statistical significance. This seems like a situation more in line with physiology of cell membranes.

Finally, the combined use of DAG and PS in PI(4)P vesicles was also tested, in an attempt to directly assay the effect of these two PKCα-effectors in the activity of Type I PI(4)P5K. As expected, the levels of $PI(4,5)P_2$ generated were raised to 647.78 ± 51.52% in the absence of PC ($p=5.99x10^{-5}$/n=5) and 372.81 ± 124.18% with PC (p=0.04/n=5). It seems likely that both the effect of DAG and PS added up to increase the performance of PI(4)P5K Iα.

Polyunsaturated DAG Is A Better Activator of PI(4)P5K Iα Expressed In Mammalian Cells Than Saturated Species

PA and DAG are related lipid species which are interconvertable by simple enzymatic reactions. PA, the major hydrolysis product of phospholipase D, can be turned into DAG by a dephosphorylation step which in living cells is catalysed by the enzyme phosphatidate phosphohydrolase (PAP). On the other hand DAG, the major hydrolysis product of phospholipase C, can be readily phosphorylated to generate PA by the enzyme diacylglycerol kinase (DGK). They are both signalling molecules with separate functions in cells. However, if these two lipids could be generated through any of the two different phospholipases and indiscriminately converted into the product of the other, an

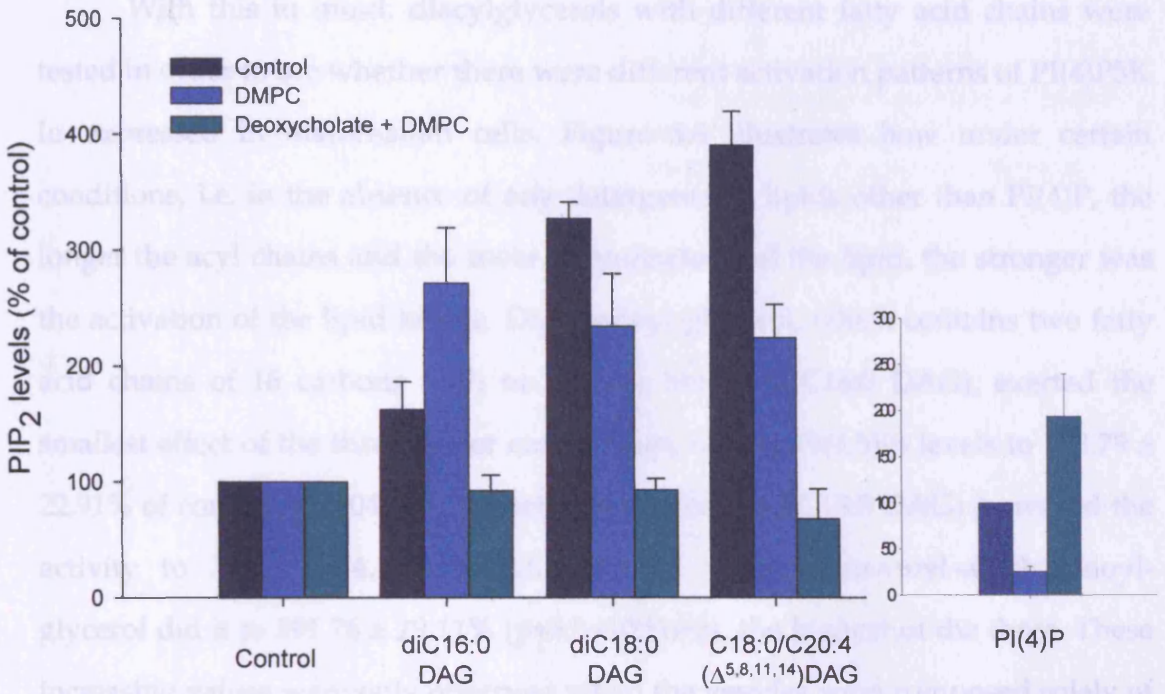


Figure 5.6 ***In vitro*** **activation of recombinant PI(4)P5K Iα expressed in Cos7 cells by different DAG mixtures.**

Recombinant Type I PI(4)P5K α was expressed in Cos7 cells as HA-tagged fusion protein, recovered on anti-HA antibody-coated agarose beads, washed and stored in frozen aliquots. A sample of the kinase was taken and diluted in kinase buffer to obtain detectable phosphorylation levels. Different lipid vesicles were tested, composed of 80 μM PI(4)P and different DAGs, in combination with 500 μM DMPC as carrier lipid, and deoxycholate (1mM). After 20 minutes at 25°C, the phosphorylation reaction was stopped by the addition of 80 μl of 10% HCl. PIP_2 levels were measured as described in the Materials and Methods section.

Data shown are mean ± S.E.M. of 3 separate experiments within which measurements were made in triplicate. For comparison, data are shown relative to the appropriate internal control since the absolute activity varied with substrate type (see insert). The statistical significance of the data was evaluated using Student's *t* test.

uncontrolled signalling loop could arise. Therefore, subtle differences in the nature of the two acyl chains that form part of the lipid might conceivably determine different substrates and origins, and thus, limit the chances of interconversion products possessing the same signalling functions as the precursor (Hodgkin *et al.*, 1998). This way, the activation of these *attenuating* enzymes may serve as a way of curtailing the signal initiated by either DAG or PA, amongst other functions.

With this in mind, diacylglycerols with different fatty acid chains were tested in order to see whether there were different activation patterns of PI(4)P5K Iα expressed in mammalian cells. Figure 5.6 illustrates how under certain conditions, i.e. in the absence of any detergents or lipids other than PI(4)P, the longer the acyl chains and the more polyunsaturated the lipid, the stronger was the activation of the lipid kinase. Dipalmitoyl-glycerol, which contains two fatty acid chains of 16 carbons with no double bonds (diC16:0 DAG), exerted the smallest effect of the three under comparison, raising $PI(4,5)P_2$ levels to 163.79 ± 22.91% of control ($p=0.049/n=3$). Distearoyl-glycerol (diC18:0 DAG) increased the activity to 327.9 ± 14.14% ($p=8.6x10^{-5}/n=3$), whereas stearoyl–arachidonoyl-glycerol did it to 391.76 ± 29.11% ($p=5.5x10^{-4}/n=3$), the highest of the three. These increasing values were only observed when the vesicles were composed solely of PI(4)P and DAG. If PC was included, the activation was similar on the three cases, between 225.2% and 271.5% ($p\leq0.039/n=3$). As expected, no activation was seen when detergents, deoxycholate in this case, and PC were included in the vesicles.

Rejection of the Possibility that the DAG-effect Is Caused by the Coprecipitation of Native DGKζ with Recombinant PI(4)P5K Iα

There are two reports in the literature on the association of PI(4)P5K with DGKζ. Tolias *et al.* (1998b) reported the formation of a complex of PI(4)P5K and DGK that was in turn capable of binding the small G protein Rac both *in vitro* and *in vivo*. They tried to identify the DGK but failed, although from the tests performed it was most likely to be DGKζ. On the other hand, Luo and colleagues (2004) have characterised the same interaction between PI(4)P5K Iα and DGKζ,

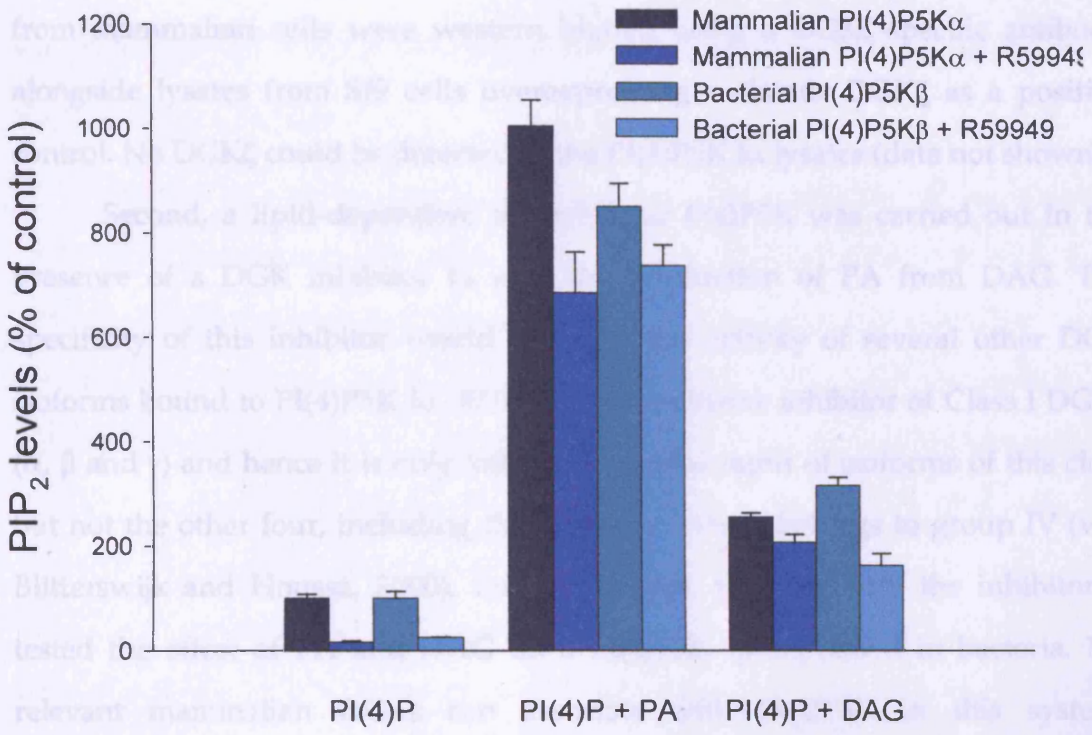


Figure 5.7 ***In vitro* activation of recombinant PI(4)P5K Iα expressed in Cos7 cells and PI(4)P5K Iβ expressed in bacteria by PA and DAG with and without the DGK inhibitor R59949.**
Recombinant PI(4)P5K Iα was expressed in Cos7 cells as HA-tagged fusion protein, recovered on anti-HA antibody-coated agarose beads; GST-tagged PI(4)P5K Iβ was expressed in bacteria, recovered on glutathione beads and eluted using reduced glutathione . A sample of each kinase was taken and diluted in kinase buffer to obtain detectable phosphorylation levels. Different PI(4)P vesicles were tested, composed of 80 μM PI(4)P and 80 μM PA (cellular extract) or diC16:0 DAG, with or without 30 μM R59949. After 20 minutes at 25°C, the phosphorylation reaction was stopped by the addition of 80 μl of 10% HCl. PIP_2 levels were measured as described in the Materials and Methods section.
Data from 1 experiment representative of another two. S.E.M. represents the deviation amongst triplicate samples. The statistical significance of the data was evaluated using Student's *t* test.

arguing that colocalisation drives the activity of PI(4)P5K by locally producing PA from DAG, especially at sites of actin remodelling such as lamellipodia.

To rule out the possibility of the activation of PI(4)P5K by DAG being a consequence of the coprecipitation of DGKζ bound to PI(4)P5K Iα expressed in mammalian cells, and therefore a real PA-activation rather than a DAG one, two different approaches were taken.

First, beads carrying overexpressed, recombinant HA-tagged PI(4)P5K Iα from mammalian cells were western blotted using a DGKζ-specific antibody alongside lysates from Sf9 cells overexpressing authentic DGKζ as a positive control. No DGKζ could be detected in the PI(4)P5K Iα lysates (data not shown).

Second, a lipid-dependent activation of P(4)P5K was carried out in the presence of a DGK inhibitor to stop the production of PA from DAG. The specificity of this inhibitor would suppress the activity of several other DGK isoforms bound to PI(4)P5K Iα. R59949 is an exclusive inhibitor of Class I DGKs (α, β and γ) and hence it is only valid to reject the input of isoforms of this class but not the other four, including the ζ isoform which belongs to group IV (van Blitterswijk and Houssa, 2000). For this matter, together with the inhibitor, I tested the effect of PA and DAG on a PI(4)P5K Iβ expressed in bacteria. No relevant mammalian DGKs can associate with PI(4)P5K in this system, definitively ruling out this possibility. Figure 5.7 shows how the inhibitor did in fact reduce the stimulation caused by DAG, and also PA. However, the levels of $PI(4,5)P_2$ in the controls were also reduced, which means that the decline was a consequence of the inhibition of the 5-kinase activity of PI(4)P5K directly. Although it cannot be seen in the figure, the stimulation caused by both PA and DAG in relation to their corresponding internal controls in the presence of inhibitor was even stronger than in its absence. $PI(4,5)P_2$ levels in the presence of PA rose to 10 ± 0.5 fold and those of DAG to 2.5 ± 0.07 fold ($p<0.0002/n=3$) when the DGK inhibitor was absent. When R59949 was included, even though the general activity decreased, the stimulation by PA in relation to its control was of 42 ± 4.8 fold and that of DAG of 12 ± 0.7 fold ($p<0.001/n=3$). Similar increases in activation were obtained with bacterial PI(4)P5K Iβ, indicating that the DAG effect is not restricted to the PI(4)P5K α isoform (see below).

There Are No Major Significant Differences In The Activation Of PI(4)P5K By PAs And DAGs With Equivalent Fatty-Acyl Chains

Finally, a side-by-side comparison of the activation of mammalian PI(4)P5K Iα and bacterial PI(4)P5K Iα, Iβ and Iγ by DAG and PA with equivalent fatty acyl chain composition was performed. The possibility of some PAs being activators of PI(4)P5Ks but not the corresponding DAGs and *vice versa* is an attractive one that could provide a mechanism to *silence* the signals arising from different pathways by metabolic interconversion. Figure 5.8 illustrates the different degrees of stimulation attained using saturated (diC16:0 and diC18:0), monounsaturated (C16:0/C18:1 and diC18:1) and polyunsaturated (C16:0/C20:4) PAs and DAGs. There were no significant differences between the PAs and DAGs with matching acyl chain compositions.

There are important differences in the behaviour of mammalian PI(4)P5Kα and bacterial PI(4)P5Kα, as well as the other two bacterial isoforms. Comparatively, the α isoform, expressed both in mammalian and bacterial cells, was always slightly less sensitive to DAGs than to the corresponding PAs. The β and γ isoforms however, in the case of unsaturated acyl chains, were more sensitive to DAGs than to PAs. In all cases, mono and polyunsaturated lipids were much more effective activators of PI(4)P5Ks than saturated ones.

The α isoform expressed in mammalian cells appeared to be more responsive to unsaturated DAGs and PAs than the bacterial one, but not to saturated forms of the lipids. The stimulation of the β and γ isoforms from bacterial origin was generally greater than that of α, especially in response to saturated DAGs where the activation was greater than the corresponding PAs, as opposed to the α isoform.

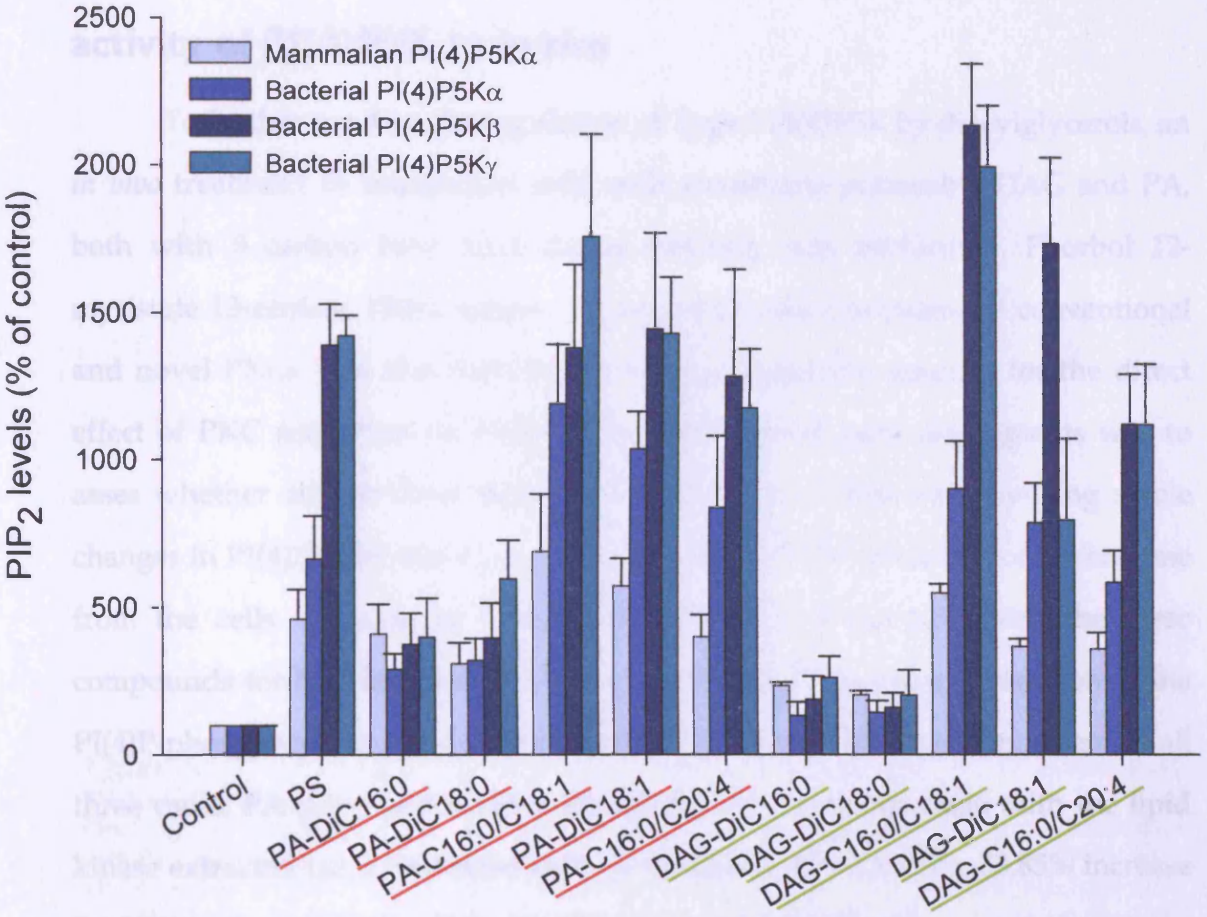


Figure 5.8 ***In vitro* activation of recombinant Type I PI(4)P5K isoforms expressed both in Cos7 cells and bacteria by different lipid mixtures.**

Recombinant Type I PI(4)P5K α was expressed in Cos7 cells as HA-tagged fusion protein, recovered on anti-HA antibody-coated agarose beads, washed and stored in frozen aliquots. A sample of the kinase was taken and diluted in kinase buffer to obtain detectable phosphorylation levels. Different lipid vesicles were tested, composed of 80 μM PI(4)P and PS, PA or DAG. After 20 minutes at 25°C, the phosphorylation reaction was stopped by the addition of 80 μl of 10% HCl. PIP2 levels were measured as described in the Materials and Methods section.

Data shown are mean ± S.E.M. of 3 separate experiments within which measurements were made in triplicate. For comparison, data are shown relative to the appropriate internal control. The statistical significance of the data was evaluated using Student's *t* test.

DAG and PMA provoke stable changes in the specific activity of PI(4)P5K Iα *in vivo*

To further confirm the regulation of Type I PI(4)P5K by diacylglycerols, an *in vivo* treatment of transfected cells with membrane permeable DAG and PA, both with 8 carbon fatty acid chains (diC8:0), was performed. Phorbol 12-myristate 13-acetate, PMA, which is a potent allosteric activator of conventional and novel PKCs was also included in the experiment to account for the direct effect of PKC activation on PI(4)P5K Iα. The aim of these experiments was to asses whether any of these three molecules were capable of provoking stable changes in PI(4)P5K Iα that would alter its activity after extraction of the enzyme from the cells. As seen in Figure 5.9, treatment of the cells with the three compounds for half an hour at 37°C and subsequent *ex vivo* examination of the PI(4)P-phosphorylating capability of PI(4)P5K Iα showed an enhancement in all three cases. PA provoked a 155 ± 12.36% increase in comparison with the lipid kinase extracted from untreated cells ($p=0.01/n=3$), DAG a 267 ± 33.85% increase ($p=0.007/n=3$) and PMA a 323 ± 31.47% increase ($p=0.002/n=3$).

The activation by DAG and PMA was stronger and statistically significant ($p\leq0.036/n=3$) in comparison with PA. The effect by DAG and PMA is supposed to be equivalent in that they both would serve as activators of PKC, although PMA is more potent for it is non-degradable by the cell machinery

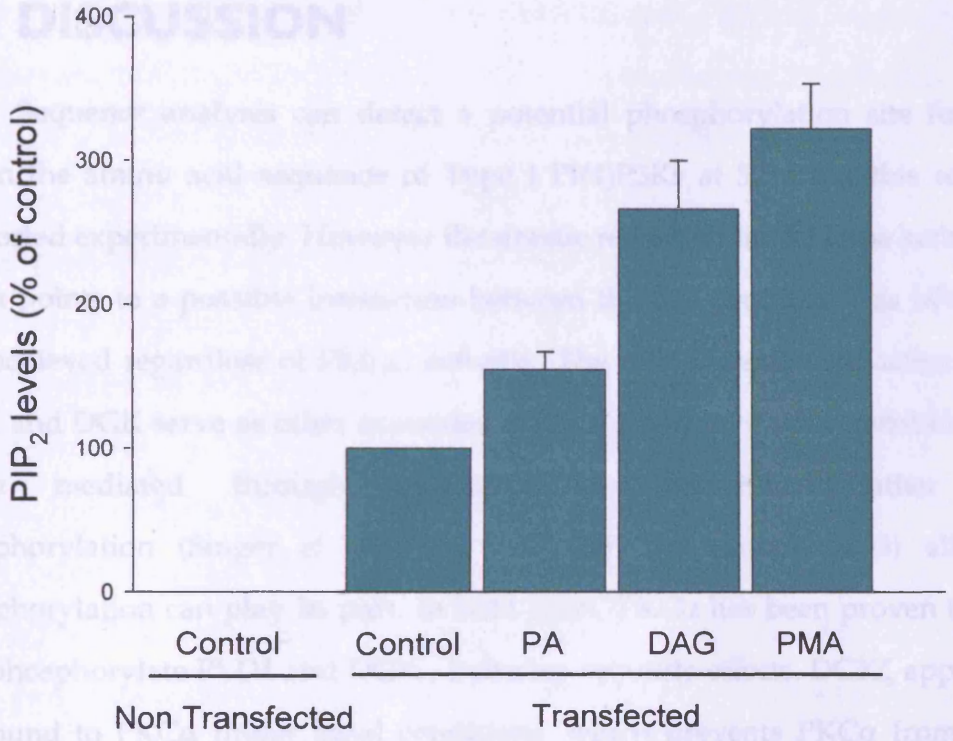


Figure 5.9 Activation of PI(4)P5K Iα by treatment of transfected cells with short chain PA/DAG/PMA *in vivo* and subsequent activity analysis *in vitro*.
Cos7 cells were transfected with GST-tagged PI(4)P5K Iα and let to express for 48 hours, then washed with PBS and incubated for 30 minutes with medium, or with medium containing diC8:0 PA (1 mM), diC8:0 DAG (1 mM) or PMA (100 nM). They were then lysed with low salt buffer, centrifuged and GST-PI(4)P5K Iα was recovered using glutathione-sepharose beads. After 3 washes in lysis buffer and one wash in kinase buffer, the beads were resuspended into 100 μl total volume, out of which 10 μl were used to test for kinase activity and the remaining 90 μl were mixed with SDS-PAGE sample buffer and resolved in a polyacrylamide gel. The levels of expressed kinase were quantified using anti-PI(4)P5K Ia antibody and these values were used to normalise the amount of PI(4)P phosphorylation under the different preincubation conditions. The control cells were transfected cells incubated only with medium, without activators, for 30 minutes.
Data shown are mean ± S.E.M. of 3 separate experiments within which measurements were made in triplicate. The statistical significance of the data was evaluated using Student's *t* test, * indicates $P<0.05$.

5.3 DISCUSSION

Sequence analysis can detect a potential phosphorylation site for PKC within the amino acid sequence of Type I PI(4)P5Ks at S214 but this was not supported experimentally. However the drastic reduction lipid kinase activity by PKCα points to a possible interaction between the two proteins. This inhibition was achieved regardless of PKCα. activity. The ATP-independent activation of PLD1 and DGK serve as other examples of the regulation of lipid metabolism by PKCα mediated through protein-protein interactions rather than phosphorylation (Singer *et al.*, 1996 and Hu and Exton., 2003) although phosphorylation can play its part. In both cases, PKCα has been proven to bind and phosphorylate PLD1 and DGK , inducing opposite effects. DGKζ appears to be bound to PKCα under basal conditions, which prevents PKCα from being activated by DAG as it is rapidly metabolised to PA by DGKζ. When an extracellular stimulus generates an increase in the level of DAG, DGKζ can no longer fully attenuate the activation of PKCα and as a consequence, the complex dissociates leaving PKCα free to phosphorylate its targets. One of these targets is DGKζ itself, which becomes inhibited upon phosphorylation by PKCα and thus, the levels of DAG can be sustained to support the activity of PKCα (Luo *et al.*, 2004). In the case of PLD1, its activation by PKCα *in vitro* has been shown to be independent of phosphorylation (Conricode *et al.*, 1992; Hammond *et al.*, 1995; Singer *et al.*, 1996; Min *et al.*, 1998; Hu and Exton, 2003). Hu and Exton (2003) and Chen and Exton (2004) have demonstrated that both PLD1 and PLD2 respectively are regulated by PKCα through a dual mechanism *in vitro* and *in vivo*. Both isoforms are activated by a fast initial protein-protein interaction with PKCα that is independent of ATP. However, a slower phosphorylation of PLD also takes place, which correlates with the inactivation of the phospholipase.

The absence of a significant effect of PKCα on PI(4)P5K Iα expressed in mammalian cells might indicate a role for post translational modification of the lipid kinase in this effect. Park *et al.* (2001) reported that PI(4)P5K Iα, β and γ expressed in NIH 3T3 cells are extracted as a phosphoprotein (S214), and the kinase responsible for it is PKA. On the other hand, Itoh *et al.* (2000) also report the existence of phospho-PI(4)P5K (again S214) although in their case they this was attributed to autophosphorylation. It is a distinct possibility that prior phosphorylation at S214 impedes PKCα and PI(4)P5K Iα interaction and is another means of integrating signals from different sources.

Nishikawa *et al.* (1998) proved the association of both Type II PI4K and Type I PI(4)P5K with the most divergent isoform of the PKC family, PKCμ or PKD. Part of their study was based on the coprecipitation of PI 4-kinase or PI(4)P 5-kinase activity together with different isoforms of PKC overexpressed in Cos7 cells. No PI(4)P5K-activity could be detected associated with PKCα. Once again, this assay does not rule out the possibility of a prior phosphorylation of PI(4)P5K by PI(4)P5K, PKA, PKC or another kinase which could be impeding the association.

The fact that the observed inhibition takes place regardless of the presence of ATP and that, when extracted from mammalian cells, this interaction does not take place could point at a possible regulation similar to that of DGKζ. Conceivably, PKCα could inhibit PI(4)P5Kα by direct protein contact which is relieved upon auto or trans phosphorylation of the lipid kinase.

The approach that was taken to determine any existent interplay between PKCα and PI(4)P5K was perhaps not exhaustive. Similarly straight-forward complimentary approaches include the detection of actual phosphorylation of the lipid kinase by PKCα using radioactive phosphate incorporation and

coexpression/coprecipitation studies to demonstrate a physical association of the two kinases.

Confirmation of the effects of the two lipid-activators PA and PS on PI(4)P5K was obtained and interestingly this was sensitive to the presence of combinations of lipids and detergents. If these results are compared with the literature describing the effect of PA on Type I PI(4)P5K then fundamental differences in the activation of the kinase are found. Moritz *et al.* in 1992 (using PI(4)P5K from bovine brain membranes), Jenkins *et al.* in 1994 (using PI(4)P5K from bovine erythrocyte membranes) and Ishiahara *et al.* in 1996 (using recombinant HA-tagged PI(4)P5K expressed in Cos7 cells), all used 0.1% Triton X-100 to generate substrate vesicles. All report different levels of activatio: 20, 8-15 and 10-fold respectively. Jenkins *et al.* also tested the activation by PA in the absence of Triton X-100, reporting a smaller activation of 3-fold. Jones D.R. *et al.* (2000) also excluded the detergent from the vesicles, arguing that it inhibits the basal activity of the lipid kinase (an observation that is not made in my system, see inset Figure 5.5), and reported a more modest 2-fold activation. These last two reports are more in accord with my results. Jones D.H. *et al.* also studied the activation of PI(4)P5K in PI(4)P/PA (1:1) or PC/PI(4)P/PA (10:1:1) vesicles, concluding that the presence of PC in their vesicles inhibit the otherwise 2-fold stimulation of Type I PI(4)P5K by PA. Again, this is not completely true in my system even though an important reduction is observed. In this last case, the differences may be due to the different mole fraction lipid ratios in the vesicles.

Far more interesting is the novel discovery of the activation of PI(4)P5K by DAG. Moritz and colleagues are the only ones to ever test the effect of this lipid on PI(4)P5K but they reported no effect. This could be due to the presence of Triton X-100 in their vesicles masking the activation since this abolished

activation in my hands. However since activation by PA under those same conditions could not be achieved this implies that the detergent is not responsible for this discrepancy but rather another factor. If we consider the vast differences in activation dependent on the nature of the fatty acyl chains of the phospholipids (see Figure 5.8), we could presume that if Moritz had used short chain DAGs (it is not specified in the article), it is likely that the stimulatory effect could have passed unnoticed.

In any case, from my *in vitro* experiments it is clear that under conditions where PI(4)P5K is activated by PA or PS then DAG is also an activator. I reject the possibility of coprecipitation of a DGKζ activity, previously shown to associate with PI(4)P5K (Tolias *et al.*, 1998b; Luo *et al.*, 2004), that could be responsible for the stimulation caused by DAG. Moreover, the use of the Class I DGK-inhibitor R59949 did not directly modify the pattern of activation caused by DAG but provoked a direct inhibition of PI(4)P5K. At concentrations above 10 μM the drug has non-specific effects on overall lipid and protein metabolism (Palicz *et al.*, 2001). The definitive test was to use PI(4)P5K expressed in bacteria which do not contain soluble DAG-kinase enzyme. In this case the effect of DAG was still present confirming that it is an intrinsic property of the enzyme rather than an artefact. All three isoforms of the lipid kinase expressed in bacteria were responsive to the presence of both PA and DAG.

Comparison of the fatty acyl dependence of PA and DAG activation of PI(4,)P5K allowed investigation of the likely sources of DAG in any cellular activation through this route in cells. 30 to 80% of cellular phosphoinositide is typically the *sn*-1-stearoyl-2-archidonoyl species. On the other hand, phosphatidylcholine predominantly contains saturated and monounsaturated fatty acids (Hodgkin *et al.*, 1998). DAG accumulation in stimulated cells is often biphasic showing a rapid rise in polyunsaturated DAGs followed by a slower

sustained phase of monounsaturated DAG accumulation alongside smaller amounts of poly-DAGs (polyunsaturated DAGs). During the initial phase, DAG is produced from the hydrolysis of $PI(4,5)P_2$ by PLC whereas that of the sustained phase is originated by PC-hydrolysis via PLD activation with subsequent dephosphorylation of PA by phosphatidate phosphohydrolase. The same applies to the generation of PAs in stimulated cells. Few stimuli activate PIP_2-PLC or PLD without simultaneously activating the other phospholipase. The extent and duration of the accumulation of the lipid products of PLC and PLD activity depends on the stimulus and receptor; bombesin desensitises rapidly within minutes and thus $PI(4,5)P_2$ or PC hydrolysis finishes rapidly while cholecystokinin (CCK) or muscarinic cholinergic stimuli desensitise much more slowly, allowing poly-DAGs to accumulate for much longer due to a persistent PLC-activation (Hodgkin *et al.*, 1998).

It is thought that it is only the polyunsaturated DAGs from PLC or the monounsaturated PAs, generated by PLD that have biological functions. Hence, interconversion of poly-DAG into poly-PA or saturated/monounsaturated PA onto the corresponding DAG would serve as a method of functional inactivation of the signal (Pettitt *et al.*, 1997; Hodgkin *et al.*, 1998; Wakelam, 1998). However I find no remarkable differences in the effects of corresponding pairs of acyl-species of DAG and PA on PI(4)P5K. Thus in contrast to signalling to PKCs and Munc-13 phosphorylation of DAG lipid by DGKs would not serve to halt the stimulation of $PI(4,5)P_2$ synthesis.

Amongst PAs and DAGs from PC-hydrolysis, there are differences amongst acyl-species principally in the effects of saturated and monounsaturated species. Saturated PAs are relatively good activators of the lipid kinase, whereas saturated DAGs are poor. Monounsaturated PAs are very strong activators of PI(4)P5K, whereas monounsaturated DAGs are less good in

the case of the Iα isoform and far better in the case of Iβ and Iγ PI(4)P5K. PS is also a better activator of these two isoforms than it is of PI(4)P5K Iα. Both murine PI(4)P5K Iβ and Iγ are the two isoforms thought to be located at the plasma membrane whereas the α isoform is thought to be located at the Golgi. This experiment demonstrates the existence of differential responsiveness to lipids by the distinct isoforms *in vitro*. Regarding saturated lipids, this could be the only way of achieving activation following PLD activation while its conversion into DAG could attenuate the signal. Nonetheless, when measuring the different fatty acids present in PA derived from PC, saturated and monounsaturated species seem to generally similar. But considering the extremely divergent effects of the two, it is conceivable that further separation of the functions of these two fatty-acyl chain species could take place within cells, allowing for further differential regulation. When PC is included in the vesicles these differences tend to disappear and so care should be taken before drawing definitive conclusions. The behaviour *in vivo* could be far from that depicted by these results.

Finally, I tested whether the activation of PKC within cells had a direct effect on PI(4)P5K that could modulate its activity once extracted from the cells, implying a permanent modification of the enzyme. The addition to cells of permeable DAG serves as a means of mimicking an external signal through PLC. The experiment proved that the addition of either DAG or PMA (a type of phorbol ester that mimics DAG-action by binding the C1 domain of cPKCs and nPKCs) provoked some stable change in the lipid kinase increasing its intrinsic activity. Park *et al.* have previously reported phosphorylation of Type I PI(4)P5K by PKA, with the consequent attenuation of its activity, and that PKC provoked a decrease in the phosphorylation, increasing its activity. Dephosphorylation was

mediated by PP1 phosphatase in response to PKC activation. My results are in accord with this model.

Cell permeable PA was also capable of altering the activity of the lipid kinase albeit much less efficiently. This result would confirm that obtained by Jones D. R. *et al.*, who also reported such an effect after treatment of transfected cells with diC8:0 PA, hence allegedly demonstrating an *in vivo* activation of PI(4)P5K Iα by PA. Alternatively PA may promote the activation of other enzymes that would permanently alter the activation state of the lipid kinase by methods such as phosphorylation or dephosphorylation. In addition to PKC that might be activated if PA was dephosphorylated to DAG several PA-dependent protein kinases have been identified. In neutrophils, PA generation provokes Ser and Tyr phosphorylation of the $p47^{phox}$ component of the NADPH complex (Waite *et al.*, 1997), in HL60 cells Tyr phosphorylation of a 100-115 kDa protein (Ohguchi *et al.*, 1997), and in 3T3-L1 cells Tyr phosphorylation of the 44/47-kDa MAP kinases (Siddiqui *et al.*, 1995), amongst other activities. These PA-dependent effects give PLD an intermediary role other than its activation being simply a consequence of PKC activation downstream of PLC activation.

Even though a conclusive effect of PKCα on PI(4)P5K could not be demonstrated with the approach described in this thesis, a further more relevant role for DAG in the control of the activity of this lipid kinase by a dual mechanism is impled. This is important because in most models of signalling to PI(4)P5K assume that PA is exclusively the lipid responsible for the feedback activation of the PIP kinase (see Figure 1.4). A revised model must include both PKC-dependent and -independent PI(4)P5K activation by DAG.

It is conceivable that both PA and DAG could work as a coupled pair and both have similar properties in terms of PI(4)P5K activation. The generation of DAG or PA as a consequence of stimulation of surface receptors is generally

linked to situations where the sustained synthesis of $PI(4,5)P_2$ is also critical. This is because, the second messengers DAG, IP_3 or PIP_3, are produced from $PI(4,5)P_2$ but also this lipid anchors a plethora of proteins involved in vesicle trafficking or actin remodelling, amongst other processes, that generally accompany receptor stimulation. Therefore, an effective, potent and rapid mechanism for $PI(4,5)P_2$ replenishment and overproduction, necessary to support simultaneous processes, has to be triggered as soon as consumption starts. DAG is the direct hydrolysis product of $PI(4,5)P_2$ and so represents a more direct stimulus to replenishment than the later generation of PA by DGK or following PLD-activation by PKC. A direct effect on PI(4)P5K reinforced by an activating dephosphorylation achieved through PKC/PP1 would serve as a quick and very powerful mechanism to increase $PI(4,5)P_2$ regeneration during the initial phase of DAG-production (see Figure 5.10).

Recently Nielssen *et al.* (2004) found that in hepatocytes, certain GPCR-agonists provoke a prolonged synthesis of DAG through $PI(4,5)P_2$ hydrolysis by PLC without a contribution from PLD. Upon vasopresin stimulation the level of PIP_2 declined, but only transiently, while increases in IP_3 and DAG mass were sustained, suggesting that efficient resynthesis of PIP_2 allows sustained PLC activity. The use of wortmannin (to block PI(4)P and $PI(4,5)P_2$ synthesis) impaired this accumulation. Therefore, as no PA-driven stimulation of PI(4)P5K could be taking place to stimulate the regeneration of the hydrolysed PIP_2, the activation of PI(4)P5K by DAG could effectively and rapidly support that resynthesis, necessary for the prolonged accumulation of DAG in these systems. These data would clearly support the positive feedback model presented on Figure 5.10.

Another important conclusion is that factors like lipid-vesicle composition, the nature of the fatty-acyl chains, the presence of detergents and/or carrier lipids and the origin of the recombinant enzymes can dramatically influence results. Consequently, conclusions must be drawn cautiously and preferably after comparing all these variables and their differential effects.

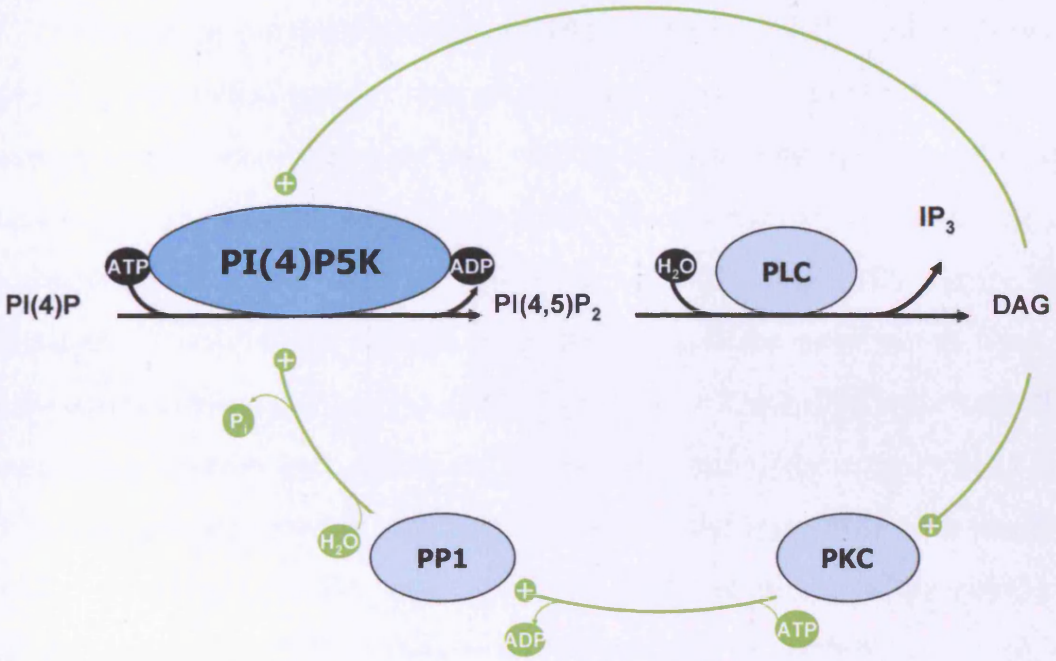


Figure 5.10 **Direct positive feedback regulation of PI(4)P5K by DAG.**
The dual feedback activation of PI(4)P5K by DAG, through direct stimulation of PI(4)P5K-activity and through activation of c/nPKCs that can indirectly provoke an increase in PI(4)P5K-activity by dephosphorylation of the lipid kinase by menas of the phosphatase PP1.

Chapter Six

Regulation of PI3K p110γ by ARF Proteins

6.1 INTRODUCTION

In this final chapter I made an attempt to characterise a novel mechanism of regulation of the production of $PI(3,4,5)P_3$ in cells by a completely ignored group of small GTPases: ADP-ribosylation factors. This approach was undertaken after the discovery of the existence of variations in the levels of this polyphosphoinositide within permeabilised HL60 cells upon readdition of members of this group of GTPases.

There is no previous evidence in the literature of direct interplay between this group of small G proteins and phosphatidylinositol 3-kinases. Class I PI3Ks have previously been shown to be subject to regulation by members of several related groups of GTPases. Gβγ subunits of heterotrimeric G proteins are responsible for the activation of the sole member of Class IB PI3Ks upon their dissociation from the Gα subunit, but also of one of the members of Class IA PI3Ks, the β isoform (Maier *et al.*, 1999). Ras binds to Class I PI3Ks through their Ras-binding domain and stimulates the level of lipid kinase activity of at least p110α and p110γ (Vanhaesebroeck *et al.*, 2001). In the case of the Rho family of small G proteins, both Rac and Cdc42 can bind and stimulate the activity of PI3Kα (Tolias *et al.*, 1995). PI3Kβ can also be activated by Rab5, a member of Rab family of small G proteins (Kurosu and Katada, 2001).

The discovery of an interaction between ARF proteins and some members of Class I PI3Ks adds complexity to the already intricate mechanisms that regulate the generation of this polyphosphoinositide in biological systems and the functions that depend on it, albeit at the same time, it opens an exciting line of study that needs further elucidation to reach a better understanding of such processes.

6.2 RESULTS

Both ARF1 and ARF6 Promote The Synthesis Of PI(3,4,5)P_3 In Permeabilised HL60 Cells

While performing permeabilised HL60 cells experiments where the ability of exogenously added ARF proteins to promote PI(4,5)P_2 synthesis was studied, a fourth spot on the TLC plates used to analyse the lipid contents of the cells appeared to change upon addition of ARF proteins and GTPγS. This spot corresponded to PIP_3, as seen by comigration studies, and figure 6.1 shows its variation with time. GTPγS on its own appeared capable of increasing the production of this polyphosphoinositide marginally, between 1.22 and 1.32-fold compared to the control over 5 and 20 minutes of the time course (no statistical significance). This probably indicates the activation of residual GTPγS-responsive GTPases inside the cells that would have an effect on the production of PIP_3 regardless of the readdition of further proteins. After 10 minutes, the maximal activation with mARF1 was attained, a 1.98-fold increase of the unstimulated control ($p<0.001/n=7$) (1.41-fold over of GTPγS) and at 15 minutes with mARF6, 1.63-fold activation ($p=0.02/n=7$) (1.36-fold of GTPγS). In both cases, the increase in PIP_3 diminished after 20 minutes to levels similar to those with GTPγS (1.32-fold of control), 1.48-fold for mARF1 and 1.59-fold for mARF6 (no statistical significance). This shows that the effect of both myristoylated ARFs in promoting PIP_3 formation was only transient and was attenuated after 20 minutes of incubation. This is to be anticipated since PIP_3-degrading enzymes such as the phosphoinositide phosphatases SHIP and PTEN which dephosphorylates PI(3,4,5)P_3 to generate PI(3,4)P_2 and PI(4,5)P_2, were likely to still be active (Damen *et al.*, 1996; Nakashima *et al.*, 2000)

Comparison of the PIP_2 and PIP_3 generation curves allows direct comparison of the different polyphosphoinositide-synthesis patterns. PIP_3 levels in response to ARF1 rise rapidly from the start of the reaction and reach higher levels than those with ARF6. This is in direct contrast to the situation with PIP_2 where ARF6 promoted a faster and remarkably higher synthesis than ARF1.

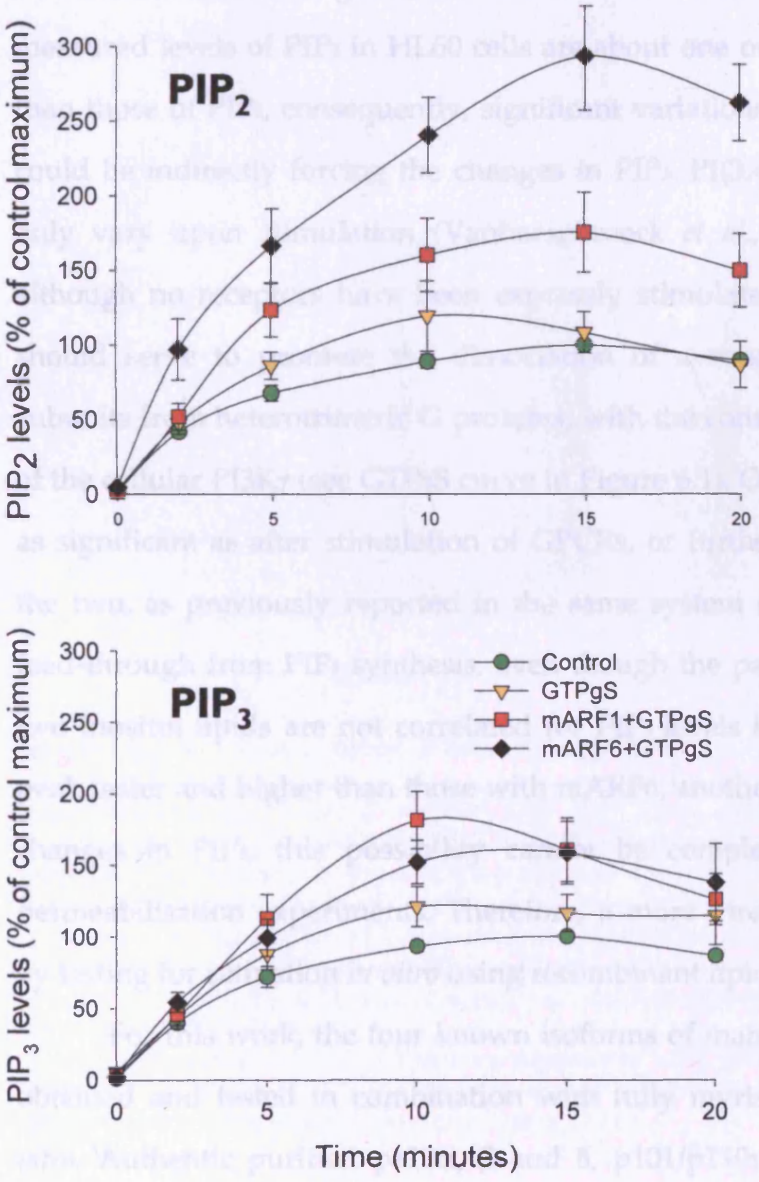


Figure 6.1 Effect of the readdition of recombinant 100% myristoylated ARF1 or ARF6 and GTPγS on PIP_3 production on permeabilised HL60 cells. After 10 minutes of permeabilisation with streptolysin O (0.4 IU/ml) at 37°C, cytosol-depleted HL60 cells were recovered by centrifugation and resuspended in buffer containing 50 μM MgATP/[γ-^{32}P]ATP final concentration (added last to start the reaction), 1 μM ARF1 or ARF6 and 10 μM GTPγS. Reactions were run at 37°C and sampled at each time point by removal of 3x50 μl identical triplicate samples of each reaction mixture and transferred into new tubes containing 80 μl of 10% (v/v) HCl. Lipid levels were measured as described in the Materials and Methods section. Data shown are mean ± S.E.M. of 7 separate experiments within which measurements were made in triplicate. Data have been plotted relative to the maximum value of the control curve. The statistical significance of the data was evaluated using Student's *t* test.

ARF Proteins Inhibit p85/p110β

A direct reading of the previous results might imply the activation of one or more isoforms of PI(3,4,5)P_3-synthesising enzymes and/or the inhibition of a PI(3,4,5)P_3-phosphatase i.e. Type I PI3Ks and SHIP/PTEN respectively. It is probable that the observed increase in PIP_3 levels following the addition of ARF proteins was purely a consequence of the increase in the levels of PIP_2, or an indirect effect on PIP_3 generation rather than a direct activation of PI3Ks. The measured levels of PIP_3 in HL60 cells are about one order of magnitude smaller than those of PIP_2, consequently, significant variations in the generation of PIP_2 could be indirectly forcing the changes in PIP_3. PI(3,4,5)P_3 levels are known to only vary upon stimulation (Vanhaesebroeck *et al.*, 2001), and in this case, although no receptors have been expressly stimulated, the addition of GTPγS should serve to promote the dissociation of a considerable number of Gβγ subunits from heterotrimeric G proteins, with the consequent activation of some of the cellular PI3Kγ (see GTPγS curve in Figure 6.1). Of course, this might not be as significant as after stimulation of GPCRs, or further more, a combination of the two, as previously reported in the same system (Kular *et al.*, 1997). As for feed-through from PIP_2 synthesis, even though the patterns of increment of the two inositol lipids are not correlated for PIP_3 levels in the presence of mARF1 peak faster and higher than those with mARF6, another contrast to the case with changes in PIP_2, this possibility cannot be completely ruled out from the permeabilisation experiments. Therefore, a more direct approach was pursued by testing for activation *in vitro* using recombinant lipid kinase proteins.

For this work, the four known isoforms of mammalian Type I PI3K were obtained and tested in combination with fully myristoylated ARF proteins *in vitro*. Authentic purified p110α, β and δ, p101/p110γ protein (crystallographic

grade) and monomeric p110γ were obtained from from Prof Mike Waterfield (Ludwig Institute for Cancer Research, UCL, London), Dr Len Stephens (Babraham Institute, Cambridge) and Prof Peter Shepherd (Department of Biochemistry, UCL, London), respectively. Given the small sample size, their authentic validated catalytic activity and their reliable provenance, the purity of these enzymes was not checked further. Figure 6.2 illustrates the effect of both ARF1 and ARF6 on the activity of the three Type IA PI3K isozymes α, β and δ.

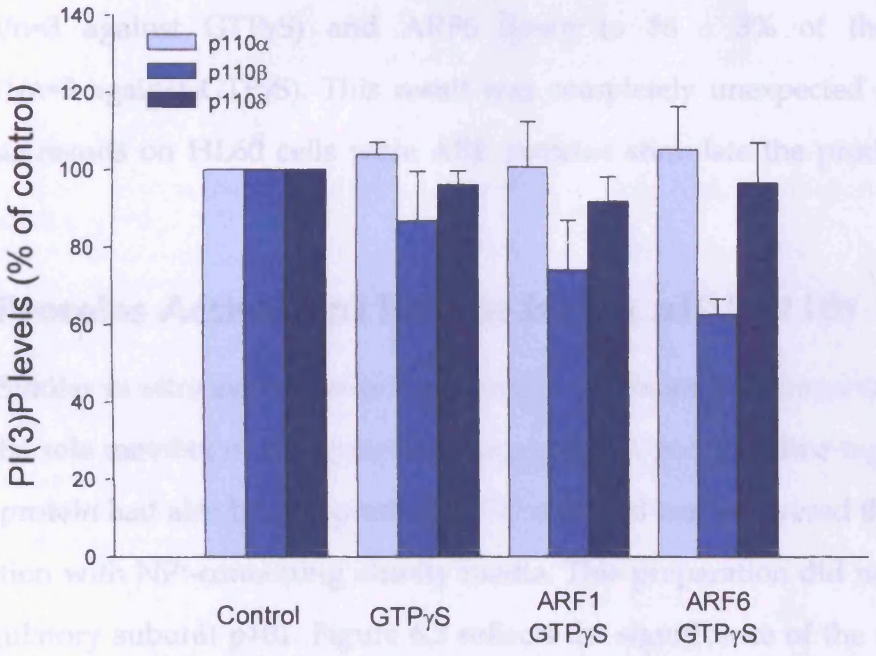


Figure 6.2 ***In vitro* effect of recombinant 100% myristoylated ARF1 and ARF6 on recombinant p110α, β and δ.**

Recombinant p110α, β and δ together with p85α were expressed in Sf9 insect cells and immunoprecipitated using anti-p85-N-SH2 antibody-coated protein A agarose beads, washed and stored in frozen aliquots. A sample of the kinase was taken and diluted in kinase buffer to obtain detectable phosphorylation levels. The kinase was mixed with final 0.2 mg/ml PI, 90 μM MgATP, 5 μCi/reaction [γ-^{32}P]ATP, 10 μM GTPγS and 1 μM recombinant ARF1 or ARF6. The samples were preincubated in ice for 40 minutes and after 30 minutes at 25°C, the phosphorylation reaction was stopped by the addition of 80 μl of 10% (v/v) HCl. PI(3)P levels were measured as described in the Materials and Methods section.

Data shown are mean ± S.E.M. of 3 separate experiments within which measurements were made in triplicate. For comparison, data are shown relative to the appropriate internal control. The statistical significance of the data was evaluated using Student's *t* test.

The three p110 catalytic subunits had been expressed in Sf9 cells together with the p85α regulatory subunit; the heterodimers were recovered on beads coated with polyclonal anti-p85 antibody, raised against the N-terminal SH2 domain. The assays were performed with the enzyme bound to the beads. In the case of the α and δ isoform there was no significant change in activity if compared with the control samples or with GTPγS. However, in the case of the β isoform, the presence of both ARF1 and ARF6 provoked a decrease in the levels of PI(3)P generated. ARF1 reduced the amount of PI(3)P to 69 ± 8% of the control ($p<0.05/n=3$ against GTPγS) and ARF6 down to 58 ± 3% of the control ($p=0.001/n=3$ against GTPγS). This result was completely unexpected given the previous results on HL60 cells were ARF proteins stimulate the production of PI(3)P.

ARF Proteins Activate p110γ But Inhibit p101/p110γ

Similar *in vitro* experiments were carried out using the remaining Type I PI3K, the sole member of the Type IB group, p110γ. A polyhistidine-tagged form of this protein had also been expressed in Sf9 cells and was recovered through its interaction with Ni^{2+}-containing affinity media. This preparation did not contain the regulatory subunit p101. Figure 6.3 reflects the significance of the activation of this isoform by both ARFs. Both ARF1 and ARF6 in the absence of GTPγS were capable of stimulating the generation of PI(3)P to 247 ± 16% and 245 ± 22% of control respectively ($p\leq0.0018/n=5$). The inclusion of GTPγS increased the amount of PI(3)P quite modestly where it was not present, up to 284 ± 17% and 339 ± 49% of control; nevertheless, this small increase was also forced by GTPγS on its own in the absence of ARF proteins, up to around 140%. This implies that such effect in the presence of ARF proteins is most certainly not due to the added GTPases

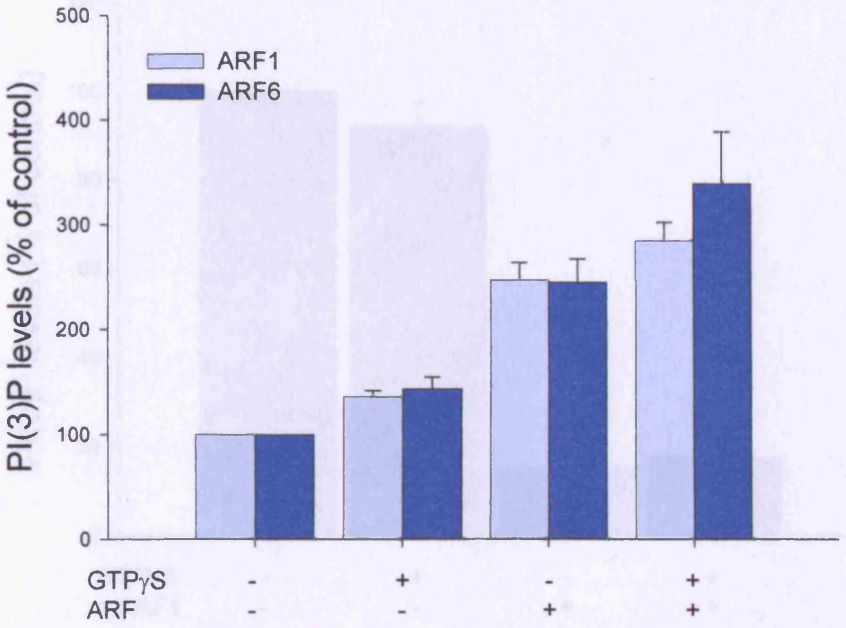


Figure 6.3 ***In vitro* effect of recombinant 100% myristoylated ARF1 and ARF6 on recombinant p110γ.**

Recombinant His-tagged p110γ was expressed in SF9 insect cells and recovered using Ni^{2+}-agarose beads, washed and stored in frozen aliquots. 10 µl of 130 nM p110γ was mixed in 50 µl final volume with final 0.2 mg/ml PI, 90 µM MgATP, 5 µCi/reaction [γ-^{32}P]ATP, 10 µM GTPγS and 1 µM recombinant ARF1 or ARF6. The samples were preincubated in ice for 40 minutes and after 30 minutes at 25°C, the phosphorylation reaction was stopped by the addition of 80 µl of 10% (v/v) HCl. PI(3)P levels were measured as described in the Materials and Methods section.

Data shown are mean ± S.E.M. of 5 separate experiments within which measurements were made in triplicate. For comparison, data are shown relative to the appropriate internal control. The statistical significance of the data was evaluated using Student's *t* test.

by GTP-loading, but due to some indirect effect of GTPγS on the lipid kinase preparation probably mediated by trace contaminants.

Moreover, since the effect on members of Type IA PI3Ks had been performed using both the catalytic and the regulatory subunits, the equivalent heterodimer of Type IB PI3K, i.e. p101 and p110γ, was obtained in order to make a direct comparison. Strikingly as seen in Figure 6.4, the stimulation of the kinase activity completely disappeared and in fact the opposite effect was attained, that is, a potent inhibition of the enzyme. ARF1 was capable of reducing the activity

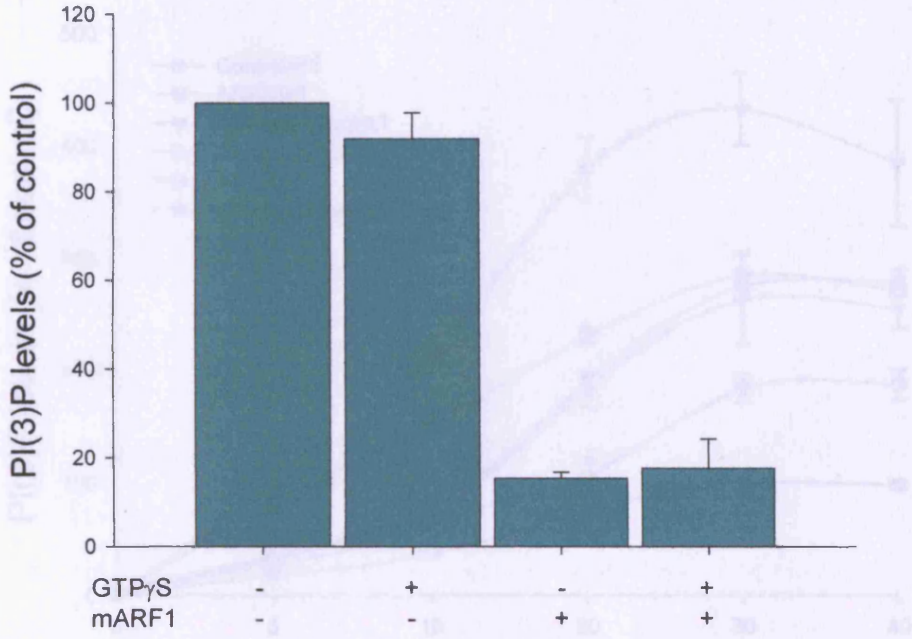


Figure 6.4 ***In vitro*** **effect of recombinant 100% myristoylated ARF1 on recombinant p101/p110γ.**

Recombinant p101/p110γ were expressed in Sf9 cells, recovered and frozen after elution from the beads. A sample of the kinase was taken and diluted in kinase buffer to obtain detectable phosphorylation levels. The kinase was mixed with final 0.2 mg/ml PI, 90 μM MgATP, 5 μCi/reaction [γ-^{32}P]ATP, 10 μM GTPγS and 1 μM recombinant ARF1. The samples were preincubated in ice for 40 minutes and after 30 minutes at 25°C, the phosphorylation reaction was stopped by the addition of 80 μl of 10% (v/v) HCl. PI(3)P levels were measured as described in the Materials and Methods section.

Data shown are mean ± S.E.M. of 3 separate experiments within which measurements were made in triplicate. The statistical significance of the data was evaluated using Student's *t* test.

of the phosphoinositide kinase down to 15 ± 1% of control in the absence of GTPγS and down to 17 ± 7% when GTPγS was included ($p \leq 0.0002/n=3$).

Testing The Effect Of GTPγS On The Activation Of p110γ By ARF Proteins *In Vitro*

ARF proteins need to be GTP-loaded in order to exert their regulatory effect onto other proteins, as it was the case of the interaction with PI(4)P5K described in Chapter 3. The fact that p110γ could be activated by both ARF1 and ARF6 in the absence of GTPγS came as a surprise, for that should not be the

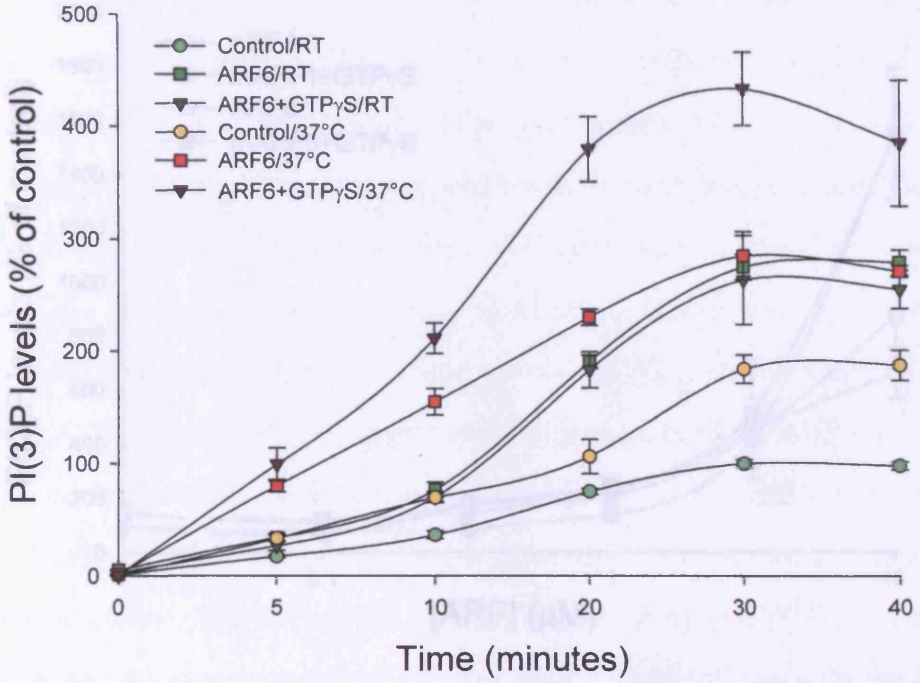


Figure 6.5 Time course of the *in vitro* effect of recombinant 100% myristoylated ARF6 on recombinant p110γ at 25°C and at 37°C.
Recombinant His-tagged p110γ was expressed in SF9 insect cells and recovered using Ni^{2+}-agarose beads, washed and stored in frozen aliquots. 10 µl of 130 nM p110γ was mixed in 50 µl final volume with final 0.2 mg/ml PI, 50 µM MgATP, 5 µCi/reaction [γ-^{32}P]ATP, 10 µM GTPγS and 1 µM ARF6 in ice. The samples were preincubated at 4°C for 60 minutes and then transferred them to 25°C or 37°C to start the phosphorylation. Reactions were sampled at each time point by removal of 3x50 µl identical triplicate samples of each reaction mixture and transferred into new tubes containing 80 µl of 10% (v/v) HCl. PI(3)P levels were measured as described in the Materials and Methods section.
Data shown are mean ± S.E.M. of triplicate samples within a single experiment. The statistical significance of the data was evaluated using Student's *t* test.

physiological scenario. For this reason, different assay conditions were tested to try to determine whether the lack of further activation in the presence of GTPγS was an artefact or a *bona fide* effect.

Temperature Dependence

Initially, the possible influence of temperature was examined. As seen in Chapter 3, preincubation of PI(4)P5K increased the stimulatory effect of ARF proteins on phosphatidylinositol 4-phosphate 5-kinase. Part of that effect may

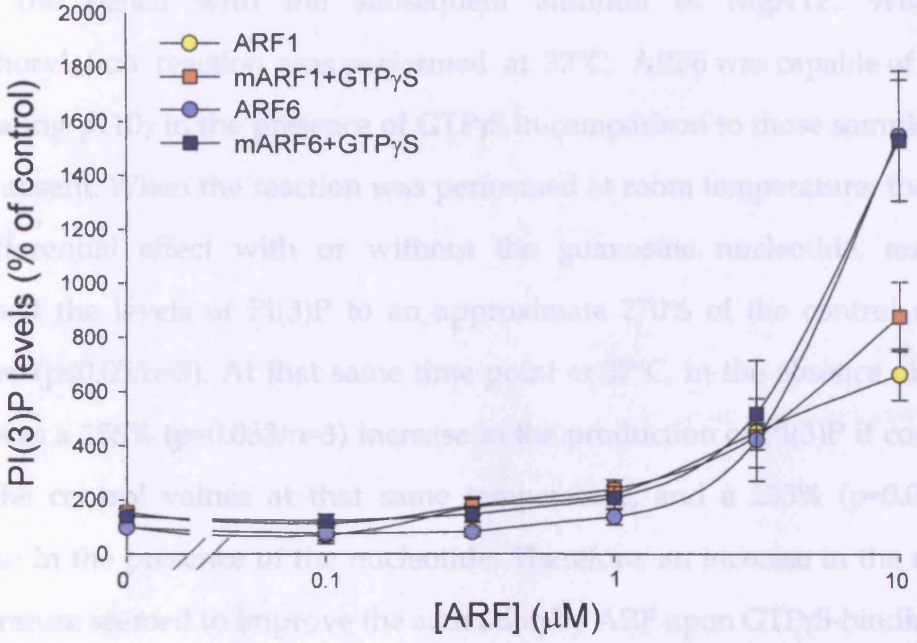


Figure 6.6 ***In vitro* effect of recombinant 100% myristoylated ARF1 and ARF6 on recombinant p110 γ.**

Recombinant His-tagged p110γ was expressed in SF9 insect cells and recovered using Ni^{2+}-agarose beads, washed and stored in frozen aliquots. 10 μl of 130 nM p110γ was mixed in 50 μl final volume with final 0.2 mg/ml PI, 50 μM MgATP, 5 μCi/reaction [γ-^{32}P]ATP, with (■) or without (●) 50 μM GTPγS and logarithmic dilutions of recombinant ARF1 and ARF6 in ice. The samples were preincubated at room temperature for 60 minutes and after 20 minutes at 37°C, the phosphorylation reaction was stopped by the addition of 80 μl of 10% (v/v) HCl. PI(3)P levels were measured as described in the Materials and Methods section.

Data shown are mean ± S.E.M. of 3 separate experiments within which measurements were made in triplicate. The statistical significance of the data was evaluated using Student's *t* test.

arise from heat-denaturation of the lipid kinase, although there is previous evidence of temperature facilitating the binding of ARF proteins to membranes as well (Haun *et al.*, 1993). However, p110γ proved not be as heat-labile as PI(4)P5K, as seen in Figure 6.5 where the control curve at 37°C runs just slightly higher than that at 25°C (all values plotted relative to the maximum point in the control line at 25°C which is the 30 minutes point). A one-hour preincubation at 4°C was performed in the absence of MgATP to allow for the components of the mixture to interact, followed by the transfer of the samples to a 37°C-water bath

or to the bench with the subsequent addition of MgATP. When the phosphorylation reaction was performed at 37°C, ARF6 was capable of further stimulating p110γ in the presence of GTPγS in comparison to those samples were it was absent. When the reaction was performed at room temperature, there was no differential effect with or without the guanosine nucleotide, and both increased the levels of PI(3)P to an approximate 270% of the control after 30 minutes ($p \leq 0.05/n=3$). At that same time point at 37°C, in the absence of GTPγS there was a 155% ($p=0.033/n=3$) increase in the production of PI(3)P if compared with the control values at that same temperature, and a 235% ($p=0.003/n=3$) increase in the presence of the nucleotide. Therefore, an increase in the reaction temperature seemed to improve the activation by ARF upon GTPγS-binding.

Figure 6.6 shows a titration curve of ARF and its influence on the activity of p110γ performed at 37°C. The stimulation on PI(3)P generation became significant at around 1 μM concentration of myristoylated ARF proteins, with little differences between the two isoforms and with no apparent influence of GTPγS on the stimulation despite the change in the temperature of the assay. The activation reached very high levels at 10 μM ARF proteins. Only at that point, ARF6 seemed to diverge on the activation of p110γ and exceed that of ARF1. In the presence of GTPγS, ARF6 yielded a 1530 ± 225% increase in PI(3)P levels ($p=0.042/n=3$) whereas ARF1 yielded a 877 ± 128% increase ($p=0.047/n=3$). Without the nucleotide the values were similar in the case of ARF6, and slightly lower in the case of ARF1 (the difference was not statistically significant). Although the previous approach pointed at a possible improvement on the activation of p110γ by ARF by means of raising the temperature of the reaction, in practical terms this did not take place, or not consistently enough to be reflected in the titration experiment.

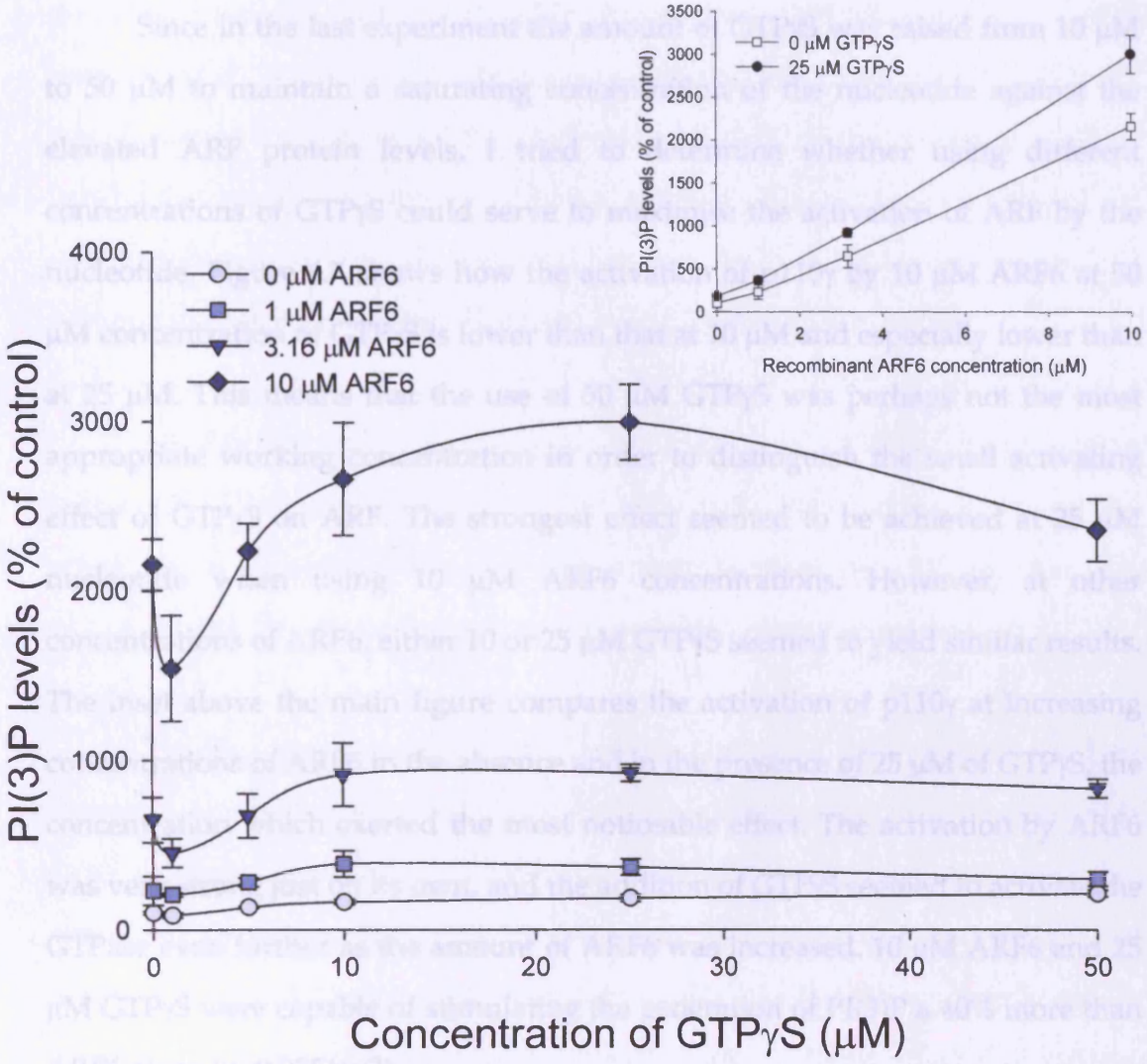


Figure 6.7 **Titration curve of the activation *in vitro* by recombinant 100% myristoylated ARF6 and GTPγS of recombinant p110γ at 37°C.**

Recombinant His-tagged p110γ was expressed in SF9 insect cells and recovered using Ni^{2+}-agarose beads, washed and stored in frozen aliquots. 10 μl of 130 nM p110γ was mixed in 50 μl final volume with final 0.2 mg/ml PI, 50 μM MgATP, 5 μCi/reaction [γ-^{32}P]ATP, different concentrations of GTPγS and of ARF6 in ice. The samples were preincubated at 4°C for 60 minutes and then transferred them to 37°C to start the phosphorylation. Reactions were stopped after 20 minutes by addition of 80 μl of 10% (v/v) HCl. PI(3)P levels were measured as described in the Materials and Methods section.

Data shown are mean ± S.E.M. of triplicate samples within a single experiment., The statistical significance of the data was evaluated using Student's *t* test.

Concentration of GTPγS

Since in the last experiment the amount of GTPγS was raised from 10 μM to 50 μM to maintain a saturating concentration of the nucleotide against the elevated ARF protein levels, I tried to determine whether using different concentrations of GTPγS could serve to maximise the activation of ARF by the nucleotide. Figure 6.7 shows how the activation of p110γ by 10 μM ARF6 at 50 μM concentration of GTPγS is lower than that at 10 μM and especially lower than at 25 μM. This means that the use of 50 μM GTPγS was perhaps not the most appropriate working concentration in order to distinguish the small activating effect of GTPγS on ARF. The strongest effect seemed to be achieved at 25 μM nucleotide when using 10 μM ARF6 concentrations. However, at other concentrations of ARF6, either 10 or 25 μM GTPγS seemed to yield similar results. The inset above the main figure compares the activation of p110γ at increasing concentrations of ARF6 in the absence and in the presence of 25 μM of GTPγS, the concentration which exerted the most noticeable effect. The activation by ARF6 was very strong just on its own, and the addition of GTPγS seemed to activate the GTPase even further as the amount of ARF6 was increased. 10 μM ARF6 and 25 μM GTPγS were capable of stimulating the generation of PI(3)P a 40% more than ARF6 alone (p=0.035/n=3).

Preloading of ARF Proteins with GTPγS

Finally, the possibility of improving the GTPγS-loading of ARF was tested using three different approaches.

The first one was by controlling the amount of Mg^{2+} present in solution to facilitate the spontaneous loading of the nucleotide. The magnesium ion found in

solutions to the structure of ARF proteins is unambiguously mandatory for GTP (or GTPγS) binding, but not for GDP (Amor *et al.*, 1994; Greasley *et al.*, 1995; Menetrey *et al*, 2000). Thus, depleting the medium of Mg^{2+} before adding the GTP may facilitate the uptake of the nucleotide by ARF as the release of GDP is usually the rate limiting step in exchange reactions. However, when this process was performed prior to the interaction between the lipid kinase and the small G protein, no improvement was observed in comparison to the control.

Another approach was made by using an **ARF-exchange factor**, specifically Cytohesin-1. This was the same tactic used when assessing the activation of phosphatidylinositol 4-phosphate 5-kinase by ARF proteins. Yet, and as occurred in the previous instance, no improvement was achieved.

Finally, the same recombinant nonmyristoylated ARF proteins used on chapter 3 to study the activation of PI(4)P5K that were 100% **preloaded** with either GDP or GppNHp were used. It needs to be highlighted once again that although this eliminates ambiguities due to GTP-loading, the proteins are not myristoylated and hence a cautious interpretation of the data needs to be made, as this is not the physiologically relevant state of these small G proteins. Nevertheless, Figure 6.8 illustrates how both GDP- and GppNHp-loaded ARF1 and ARF6 increase the production of PI(3)P by p110γ. Once again and confirming the behaviour reported when interacting with PI(4)P5K, the nonmyristoylated GDP-loaded forms of both ARF1, up to 323 ± 49% of control, and ARF6, up to 397 ± 68 ($p \leq 0.010/n=3$), are more efficient in activating p110γ than the GppNHp-loaded forms. GppNHp-loaded ARFs, increase the PI(3)P levels up to 181 ± 0% and 226 ± 18% of control respectively ($p=0.002/n=3$). The better performance of

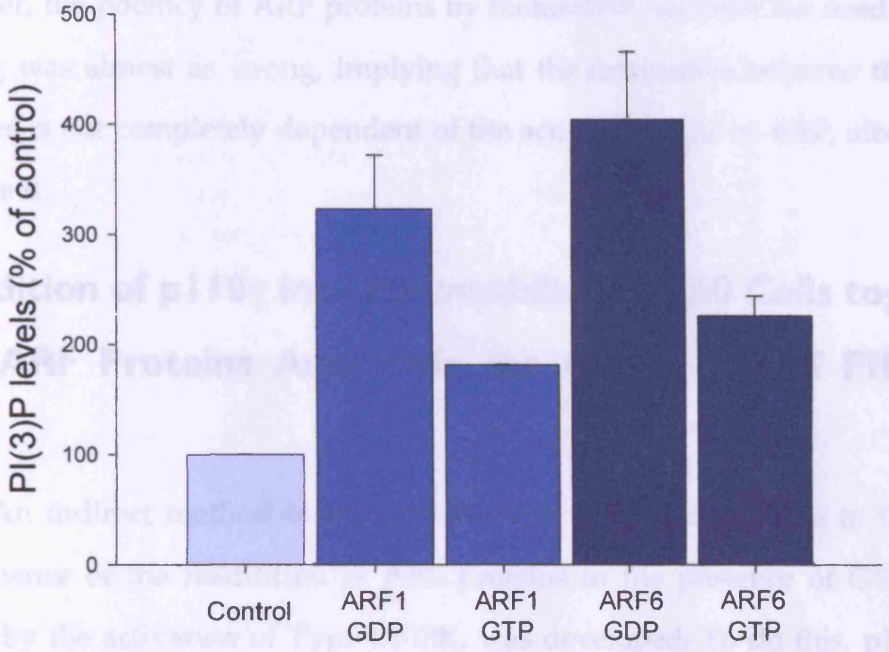


Figure 6.8 ***In vitro* activation of recombinant p110γ by recombinant preloaded nonmyristoylated GDP/GppNHp-ARF1 and ARF6.**
Recombinant His-tagged p110γ was expressed in SF9 insect cells and recovered using Ni^{2+}-agarose beads, washed and stored in frozen aliquots. 10 μl of 130 nM p110γ was mixed in 50 μl final volume with final 0.2 mg/ml PI, 90 μM MgATP, 5 μCi/reaction [γ-^{32}P]ATP and 10 μM recombinant preloaded GDP/GppNHp-ARF1 or ARF6. The samples were preincubated at 37°C for 30 minutes, the ATP added and after 20 minutes at 25°C, the phosphorylation reaction was stopped by addition of 80 μl of 10% (v/v) HCl. PI(3)P levels were measured as described in the Materials and Methods section.
Data shown are mean ± S.E.M. of 3 separate experiments within which measurements were made in triplicate. The statistical significance of the data was evaluated using Student's *t* test.

the GDP-loaded forms with both isoforms of ARF is also statistically significant ($p \leq 0.05/n=3$). In contrast to what happened when using PI(4)P5K as the effector of preloaded nonmyristoylated ARF proteins, there is no apparent preference for ARF6 over ARF1.

In conclusion, there were certain conditions where the effect of GTP (analogues) on the activation of p110γ by ARF proteins could be maximised in order to demonstrate that GTPγS could further stimulate such interaction *in vitro*.

However, the potency of ARF proteins by themselves, without the need of GTP-binding was almost as strong, implying that the interaction between these two enzymes is not completely dependent of the activation state of ARF, albeit it can improve it.

Readdition of p110γ into Permeabilised HL60 Cells together with ARF Proteins Augments the Generation of PIP_2 and PIP_3

An indirect method to confirm that the registered increase in PIP_3 as a consequence of the readdition of ARF proteins in the presence of GTPγS was caused by the activation of Type I PI3K, was developed. To do this, p110γ was introduced into the permeabilised HL60 reconstitution assay in combination with fully and nonmyristoylated ARF proteins and GTPγS. Figure 6.9 reflects the PIP_2 and PIP_3 levels generated after 10 minutes of the readdition of the components.

Looking at the *Control* data for the **PIP_3** graph, it is apparent that neither GTPγS nor PI3K were capable of significantly altering the cellular contents of PIP_3. This would mean that the mere enrichment of the cellular contents of Type IB PI3K by exogenous addition is not sufficient to increase PIP_3 levels in cells, since physiological activation of this class I PI3K is dependent on its interaction with an activator in response to a stimulus, and the presence of its regulatory subunit, p101, seems mandatory by mediating membrane recruitment (Brock *et al.*, 2003). However, when both agents were used in conjunction, PIP_3 levels rose to 135 ± 9% of control, statistically significant with respect to both control and GTPγS stimulation ($p \leq 0.023/n=3$). This could reflect a situation where addition of GTPγS could be activating some of the membrane bound heterotrimeric G

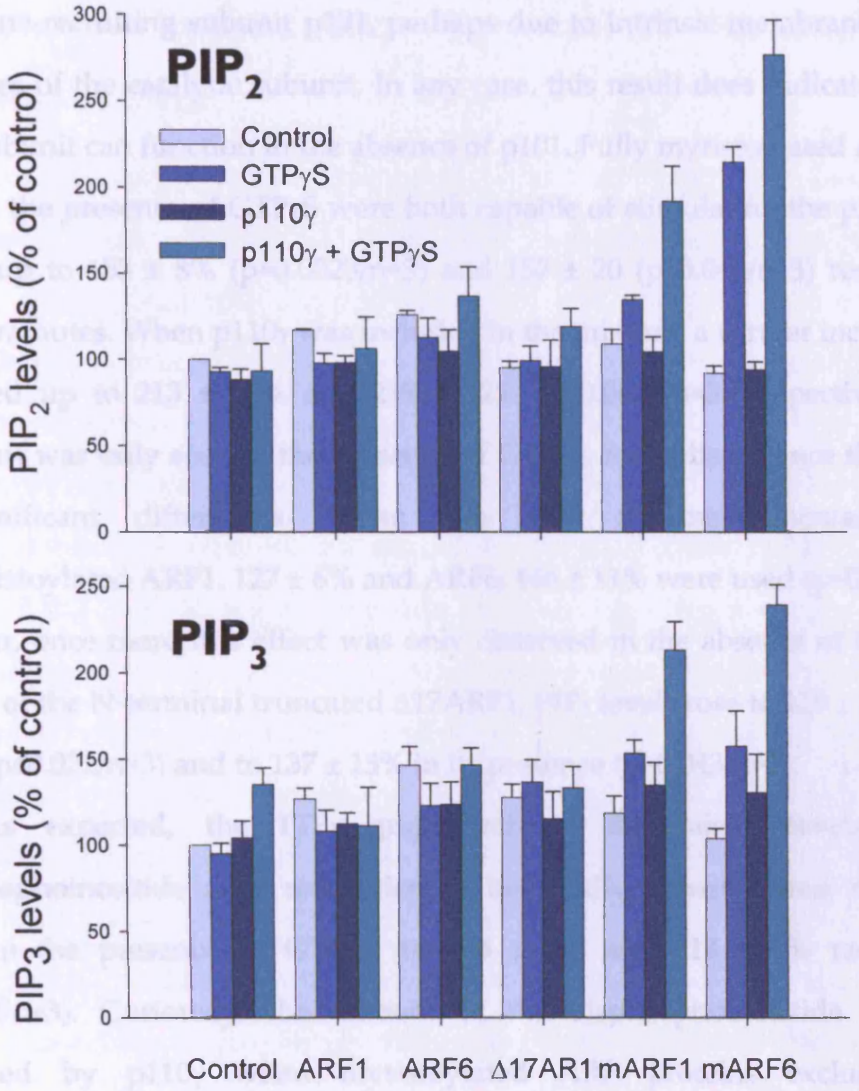


Figure 6.9 **Effect of the readdition of recombinant ARF proteins, p110γ and GTPγS on PIP_2/PIP_3 production after 10 minutes on permeabilised HL60 cells.**

After 10 minutes of permeabilisation with streptolysin O (0.4 IU/ml) at 37°C, cytosol-depleted HL60 cells were centrifuged and resuspended in buffer containing 50 μM MgATP/[γ-^{32}P]ATP final concentration (added last to start the reaction), 1 μM nonmyristoylated ARF1, Δ17ARF1 or ARF6, or fully myristoylated ARF1 or ARF6, 26 nM p110γ and 10 μM GTPγS. Reactions were run at room temperature for 10 minutes and stopped by addition of 80 μl of 10% (v/v) HCl. Lipid levels were measured as described in the Materials and Methods section.

Data shown are mean ± S.E.M. of 3 separate experiments within which measurements were made in triplicate. Data have been plotted relative to the maximum value of the control curve. The statistical significance of the data was evaluated using Student's *t* test.

proteins to release βγ subunits and these in turn stimulate the reintroduced p110γ. However, this would have to be an interaction in the absence of its

membrane-recruiting subunit p101, perhaps due to intrinsic membrane binding properties of the catalytic subunit. In any case, this result does indicate that the p110γ subunit can function in the absence of p101. Fully myristoylated ARF1 and ARF6 in the presence of GTPγS were both capable of stimulating the production of PIP_3 up to 153 ± 8% (p=0.0023/n=3) and 157 ± 20 (p=0.046/n=3) respectively after 10 minutes. When p110γ was included in the mixture, a further increase was registered up to 213 ± 16% and 239 ± 12% (p≤0.0023/n=3) respectively. This increment was only seen in the presence of GTPγS, for in its absence there were no significant differences. There was also a small increase when nonmyristoylated ARF1, 127 ± 6% and ARF6, 146 ± 11% were used (p=0.012/n=3). However, once more this effect was only observed in the absence of GTPγS. In the case of the N-terminal truncated Δ17ARF1, PIP_3 levels rose to 128 ± 8 without GTPγS (p≤0.022/n=3) and to 137 ± 13% in its presence (p=0.043/n=3).

As expected, the **PIP_2** graph reflects the raised levels of the polyphosphoinositide after readdition of both fully myristoylated ARF1 and ARF6, in the presence of GTPγS, to 135 ± 3% and 214 ± 9% respectively (p≤0.002/n=3). Curiously, the amount of the bisphosphoinositide was also influenced by p110γ when myristoylated ARF proteins exclusively in combination with GTPγS were present, further rising to 192 ± 20% (p=0.001/n=3) and 276 ± 20% (p=0.0009/n=3). This could point at an activation of PI(4)P5K by collaboration of both PIP_3 lipids and GTP-loaded ARFs, conceivably through increased ARF-GEFs activation. It was again intriguing to see that nonmyristoylated ARF1, 117 ± 1%, and ARF6, 126 ± 3% (p≤0.0006/n=3), were capable of slightly increasing the levels of PIP_2 in the absence of GTPγS, an increment that was abolished in the presence of the nucleotide. Δ17ARF1 had no effect on the levels of PIP_2.

Brefeldin A Inhibits the Migration of Neutrophils in Response to fMLP

Another approach to examine whether ARF proteins have a real involvement in the control of p110γ in a more biological context was pursued by trying to disrupt indirectly the performance of ARF in a process dependent on PI3Kγ. One such process is the migration of neutrophils towards a source of the tripeptide N-formyl-Methionyl-Leucyl-Phenylalanine (fMLP) where p110γ is known to be a key player (Rickert *et al.*, 2000). For this, the effect of brefeldin A, a known inhibitor of most of the so called "Big ARF-GEFs" (Donaldson and Jackson, 2000), was tested side-by-side with wortmannin, a extensively used PI3K inhibitor. The experimental approach allowed quantification of the relative migration of neutrophils situated in an upper chamber, separated by a cellulose nitrate filter from a lower chamber where the source of fMLP was located. In an attempt to reach the source of bacterial peptide, neutrophils migrated towards it, becoming embedded inside the filter at different depths. Counting all cells that had progressed past a given migration depth (20 μm from the top surface of the filter) indicated the degree of migration thus assaying normal chemotaxis and the disruption caused by the compounds mentioned. Figure 6.10 illustrates how the inclusion of the source of fMLP (10 nM) in the bottom chamber increased the migration of cells by 49.3 ± 2.9% and that this was insensitive to the presence of dilute DMSO ($p \leq 0.01/n=3$), the solvent vehicle used for drug additions. That value can be considered as the amount of neutrophils attracted by the peptide, rather than by randomised movement. When wortmannin was included in the upper chamber, the number of cells embedded deep in the filter was diminished to values closer to control, 111 ± 8.6%, which means an elimination of migration

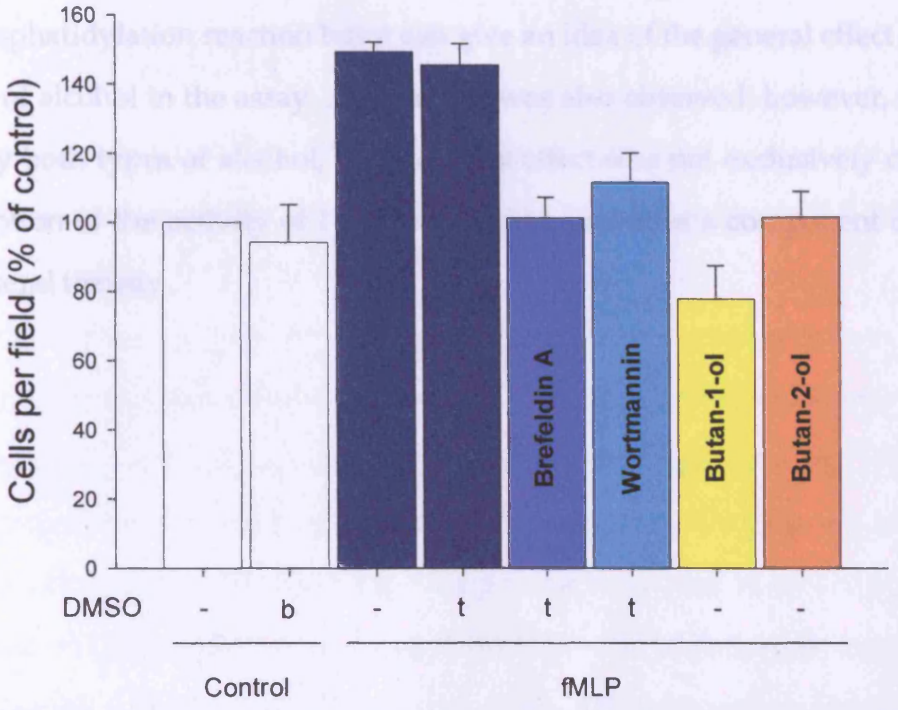


Figure 6.10 **Effect of different compounds in the migration of neutrophils towards an fMLP source.**

.Neutrophils were extracted and introduced in the top compartment of a two-chamber container where the two chambers were separated by a semipermeable membrane. Neutrophils in the top chamber were left to migrate randomly (Control) or after addition of fMLP peptide in the bottom chamber (fMLP) which should attract them towards the membrane and penetrate it to different degrees. Different compounds were tested in their ability to hinder the directional migration towards the stimulus, namely, brefeldin A, wortmannin, butano-1-ol or butan-2-ol, by their addition in the top chamber. Controls with DMSO in the bottom (b) or top (t) chamber were used to reject any influence of this compound in the migration, as brefeldin A and wortmannin are diluted in this solvent. After X minutes, the membrane was extracted, washed and the amount of neutrophils inserted into the membrane were counted across 6 different optical fields and averaged.

Data shown are mean ± S.E.M. of 3 separate experiments within which measurements were made with 6 different samples. The statistical significance of the data was evaluated using Student's *t* test.

towards fMLP (p=0.014/n=3). When Brefeldin A was used, migration was eliminated as effectively as with wortmannin, down to 101 ± 6% (p=0.002/n=3).

For comparison, PLD-dependent PA-production was inhibited (but not eliminated) with dilute primary aliphatic alcohol butan-1-ol in order to assess the involvement of the phospholipase in the process. The secondary alcohol butan-2-ol was used as a control as it does not participate in the

transphosphatidylation reaction but it can give an idea of the general effect of the presence of alcohol in the assay. A reduction was also observed; however, it was caused by both types of alcohol, therefore the effect was not exclusively caused by disruption of the activity of PLD but certainly includes a component due to more general toxicity.

6.3 DISCUSSION

The main focus of this chapter has been to establish and characterise the novel activation of one of the isoforms of Class I PI3K, PI3Kγ, by small G proteins of the ARF family.

PI3Ks have long been known to be regulated by several members of the Ras superfamily of small G proteins, as well as by Gβγ subunits of heterotrimeric G proteins. GTP-loaded Ras, but not GDP-Ras, interacts with class IA PI3Ks and farnesylation of Ras at C186 is crucial for PI3Kα activation. Ras interacts with the catalytic subunit p110, although only PI3Kα is demonstrated to be activated by this interaction (Rodriguez-Viciana *et al.*, 1994, 1996) although the sole member of Class IB, p110γ (Pacold *et al.*, 2000; Suire *et al.*, 2002) also interacts with Ras. Alternatively, Ras and possibly other small G proteins, can bind to the p85 regulatory subunit and activate PI3K in exclusive conjunction with phosphotyrosine-peptides (Jimenez *et al.*, 2002; Chan *et al.*, 2002).

Rac and Cdc42, two of the various members of the Rho family of small GTPases, are capable of interacting and activating PI3Kα (Tolias *et al.*, 1995; Bokoch *et al.*, 1996) and can bind the Rho-GAP homology domain of p85 (Zheng *et al.*, 1994). PI3Ks become activated downstream of Rac (Genot *et al.*, 2000; Rickert *et al.*, 2000), despite the fact that these small G proteins, and others such as ADP-ribosylation factors, appear to function downstream of PI3K as well. Obviously, these seeming contradictions might be reconciled if these G proteins and the kinase are part of a positive feedback loop. There is evidence showing that PI3K can activate Rac, and other small G proteins, indirectly via PI(3,4,5)P_3-sensitive GEFs (Welch *et al.*, 2003), thus the activation of PI3K by Rho GTPases is considered to be generally the consequence of a positive feedback loop that

results in an enhanced production of $PI(3,4,5)P_3$ at particular sites on the membrane. This would be the case at the leading edge of migrating neutrophils (Weiner *et al.*, 2002; Srinivasan *et al.*, 2003).

Additionally, PI3Kβ has also been found to be activated by Rab5, one of the members of the extensive Rab family, which binds to the catalytic subunit p110β (Kurosu *et al.*, 2001).

Finally, both p110γ (Stoyanov *et al.*, 1995; Stephens *et al.*, 1997; Leopoldt *et al.*, 1998) and p110β (Kurosu *et al.*, 1997; Katada *et al.*, 1999; Maier *et al.*, 1999, 2000) can also be activated by Gβγ subunits of heterotrimeric G proteins liberated upon activation of G-protein coupled receptors (GPCRs). In the case of p110β, the presence of p85 is not necessary, although its presence in combination with the binding of phospho-tyrosine peptides enhances the activation. In the case of p110γ, p101 is not necessary either for its activation by Gβγ, albeit its presence seems to sensitise PI3Kγ to phosphorylate $PI(4,5)P_2$ rather than PI (Maier *et al.*, 1999).

The appearance of a radioactive PIP_3 spot in the TLC during the HL60 reconstitution assays, which appeared to vary in response to the presence of ARF proteins, led to an in-depth study of the causes of this variation. Class I PI3Ks, the only enzymes thought to be responsible for the generation of this second messenger *in vivo* (Carpenter and Cantley, 1990) are cytosolic proteins that are recruited to membranes upon stimulation (Klippel *et al.*, 1996). Hence, permeabilisation of unstimulated cells should have depleted the cell content of at least part of the PI3Ks; as previously reported. After 10 minutes approximately 75% of the cellular PI3Kγ–content leaks out of permeabilised HL60 cells (Kular *et al.*, 1997), but even after longer permeabilisation there is some remaining kinase

within the cells that does not leak out. I assume that the process is similar with the remaining Class IA isoforms that are also present in this particular cell type and which are also cytosolic. Thus, there would still be enough PI3Ks to generate PIP_3. As seen in Figure 6.1, the increase in the levels of PIP_3 was transient and decreased after approximately 20 minutes of readdition of the different proteins however the change was not as prominent as that seen with the levels of PIP_2. This is probably foreseeable as no stimulus had been given to the cells in order to fully activate PI3K enzymes, and no inhibition of PIP_3-phosphatases, such as PTEN or SHIP, had been implemented either. Therefore the limited activation registered was probably caused by the few remaining PI3K enzymes that could become activated in the absence of stimulation and whose product became degraded after a few minutes.

Assessment of the *in vitro* activation of the diverse isoforms of Class I PI3K by ARF proteins could help establish direct activation of the kinase. Class IA enzymes were tested as heterodimers, *i.e.* the catalytic subunit p110α/β/δ together with the regulatory subunit p85α all immobilised on beads. This is essential as the catalytic subunits are extremely unstable in the absence of its regulatory adaptor (unlike p110γ, see below). Class I enzymes α and δ were not affected by the presence of ARF proteins. However, the β isoform was partially inhibited by the presence of both ARFs.

It is conceivable that an effect of ARF on the other isoforms of Class IA PI3Ks is still plausible. It has been demonstrated that p85 inhibits p110 when associated (Yu *et al.*, 1998). This inhibition is released when p85 binds, via its SH2 domain, tyrosine-phosphorylated proteins (Jimenez *et al.*, 2002) and may be enhanced by phosphorylation at Y688 (Chan *et al.*, 2002). Further investigation of

the effect of ARF proteins should include tyrosine-phosphorylated proteins or peptides to which p85 could bind, after elution from the recovery beads to fully assess this possible interaction as it happens.

On the other hand, when p110γ was tested separately from its regulatory subunit p101, a potent increase in the phosphorylation of PI was observed. Consequently, my main efforts were centred in the characterisation of this interaction. Later I tested the effect of ARF proteins in the p101/p110γ heterodimer with the intention of making a comparative analysis of its influence in relation to Class IA enzymes. Strikingly, not only did ARF not stimulate the activity of the PI3Kγ heterodimer but it strongly suppressed it. It is interesting how both heterodimeric PI3Kβ and PI3Kγ reacted in a similar manner to the presence of ARF. PI3Kβ is the only isoform of Class IA PI3Ks that can be activated by Gβγ subunits of heterotrimeric G proteins in the same way as PI3Kγ. Class IA enzymes become activated by the binding of p85 to growth factor receptors or associated proteins that become phosphorylated upon stimulation, at pYxxM motifs. The β isoform however, can also be activated by Gβγ subunits released upon activation of GPCRs (which is the typical activation mechanism of PI3Kγ) and this such activation is further enhanced by the presence of phospho-tyrosine peptides derived from RTK stimulation in the case of PI3Kβ (Kurosu *et al.*, 1997; Katada *et al.*, 1999; Maier *et al.*, 1999, 2000). It is intriguing to see that the two isoforms that can be regulated by heterotrimeric G proteins can both be inhibited by ARF when in their heterodimeric form. This further raises the question on whether monomeric p110β may also be activated by ARF, and if so, whether it has any biological relevance. Again further investigations of ARF effects on these kinases may benefit from the inclusion of Gβγ subunits in the assay.

Although it became clear from the *in vitro* p110γ activation assays that the catalytic subunit could be strongly stimulated by ARF, the fact that this activation was independent of the nucleotide-bound state of ARF was not at all expected. Variations in the temperature and duration of the preincubation, in the temperature of incubation and the concentration of GTPγS, improved GTPγS-loading or the use of fully nucleotide-loaded nonmyristoylated ARF proteins, proved to be moderately helpful, but in no instance definitive proof that GTP-ARF could activate p110γ significantly better than GDP-ARF. There is one report by Rubio *et al.* (1999) showing that post-translationally, i.e. farnesylated, modified Ras associates with p110γ independent of GTP, whereas non-farnesylated Ras only does it upon GTP binding; nevertheless, GTP-Ras forms a much tighter complex with p110γ. This behaviour is not shared by p110α, which only binds to GTP-Ras. Despite these data, there is strong evidence showing that catalytic activation of p110γ is exclusively GTP-Ras dependent (Pacold *et al.*, 2000; Suire *et al.*, 2002). It is possible that the observed GTP-independent activation by ARF is an artefactual consequence of using lipid vesicles containing excessive amounts of PI. Kirsch *et al.* (2001) presented strong evidence that membrane tethering of p110γ may involve association with anionic phospholipids and Krugmann *et al.*, (2002) in *in vitro* conditions, showed that most of PI3Kγ is already associated with lipid vesicles in the absence of Gβγ. These reports taken together could mean that p110γ, in the exclusive presence of PI, is already vesicle-bound and therefore the GTP-dependent association to membranes characteristic of ARF would have been overcome by its binding to the already lipid-associated p110γ. In a way, it would be as if the roles of the two proteins would have been inverted. Since either GDP-ARF or GTP-ARF provokes a clear increase in the activity of p110γ, an allosteric activation of the enzyme

ARFs. This seems to be a recurring behaviour of nonmyristoylated forms of ARF. When nonmyristoylated (not preloaded) ARFs were used in the permeabilisation cell reconstitution assay with p110γ, there was always a small increase in the levels of PIP_3 in the absence of GTPγS or p110γ, and also in the levels of PIP_2; this effect was suppressed upon addition of GTPγS. In chapter 3, Figures 4.14 and 4.17 also show, although more moderately, how the effect of nonmyristoylated ARF proteins on PI(4)P5K in the absence of GTPγS is always relatively higher than in its presence. These different assays highlight once again a peculiar characteristic of ARF proteins out of which a conclusion must be drawn: the use of nonmyristoylated or partially myristoylated preparations of the recombinant protein should be avoided due to the complicated and often opposing effects upon GDP- or GTP-binding. In biological terms, it is unnecessary and probably irrelevant to analyse the consequences of this odd behaviour in depth, as nonmyristoylated forms of this small GTPase do not occur in living cells. Another conclusion that can be extracted from this experiment is that in the case of p110γ there was not a marked preference for any of the two isoforms of ARF, as was already apparent from the other *in vitro* and reconstitution assays. This lack of preference throws into stark relief the selectivity of PI(4)P5K for ARF6 (Chapter 3, Figure 4.15), as they are both parallel activation assays of phosphoinositide kinases using the same recombinant ARF proteins.

Finally, I attempted to imply the involvement of these small G proteins in one of the most studied cell biological processes where PI3Kγ is involved, the migration of neutrophils. Leukocytes are capable of detectings gradients of chemoattractant, allowing them to move towards injured or inflamed tissue.

would be taking place as a consequence of their association, rather than the recruitment of the lipid kinase to membranes. In line with this, farnesylated Ras is thought to promote a conformational change in the structure of p110γ upon association and this is thought to allosterically activate the enzyme. Indeed, structural studies reveal a direct interaction of Ras with the COOH-terminal part of the catalytic domain of p110γ and a difference in the conformation of the catalytic domain between p110γ and Ras-p110γ (Pacold *et al.*, 2000). In the permeabilised-cell system however, it was obvious that PIP_3 generation, both in the presence and absence of reintroduced p110γ, was conditional to the presence of GTPγS. Hence in cells, the basal recruitment of p110γ to membranes may be minimal, and the activation by ARF dependent on its GTP-bound state and consequent membrane relocation of the two proteins. What cannot be inferred from the results in this system is whether ARF is solely responsible for the increased p110γ membrane relocation, as supposed from the higher yield of PIP_3 obtained, or whether another parallel mechanism is facilitating the colocalisation of the two proteins on the membrane. GTPγS on its own does not stimulate a significant increase in PI(3,4,5)P_3 levels unless accompanied by myristoylated ARF, therefore the activation of Gβγ subunits by readdition of GTPγS is not sufficient to activate p110γ. Ras does not drive membrane translocation of PI3Kγ either *in vivo* or *in vitro* (Suire *et al.*, 2002). This might be the case with ARF as well, although important differences in the functional behaviours of these two small G proteins could allow for this mechanism.

The use of fully GDP- or GppNHp-loaded nonmyristoylated ARFs did not reveal the GTP-dependent activation of p110γ. In accordance with effects on PI(4)P5K, but in an even more pronounced manner, GDP-loaded ARFs stimulated the activity of p110γ almost twice as potently as GppNHp-loaded

Neutrophilic leukocytes orient their polarity in response to such a gradient and migrate in the right direction up the concentration gradient. Chemoattractants such as fMLP stimulate GPCRs, which upon activation of a heterotrimeric G_i protein, release the Gβγ subunit. Amongst many downstream effectors, which include Rho GTPases, MAP kinases, PKCζ, cytosolic tyrosine kinases and cPLA2, there is a key role for PI3Ks. PI3K inhibitors reduce chemotaxis of four types of leukocytes, neutrophils, macrophages, T cells and natural killer cells (Rickert *et al.*, 2000). Use of PI3Kγ$^{-/-}$ mice has revealed that the chemotaxis of neutrophils is severely impaired in the absence of PI3Kγ, both *in vitro* and *in vivo* (Li *et al.*, 2000; Sasaki *et al.*, 2000; Hirsch *et al.*, 2000; Hannigan *et al.*, 2002). However, little effect is observed on random movement of these cells suggesting that PI3Kγ regulates chemotaxis primarily by controlling the direction of cell migration and the intracellular colocalisation of Akt and F-actin to the leading edge (Hannigan *et al.*, 2002). In neutrophils, PI3K activity is required for attractants to activate two GTPases directly responsible for cytoskeletal changes leading to cell movement, Rac and Cdc42 (Benard et al., 1999). Asymmetric accumulation of PI(3,4,5)P_3 and actin at the leading edge, with the consequent formation of pseudopods, depends upon a positive feedback loop in which accumulation of the lipid activates Rho GTPases, whereas activated Rho GTPases and (probably) polymerised actin in turn increase accumulation of the lipid (Wang et al., 2002; Weiner et al., 2002).

Recognising that some ARF-GEFs can be inhibited by Brefeldin A (BFA) then tbis reagent can indicate the involvement of ARF proteins in the migration process. It is a limited result, however, as BFA exclusively inhibits the BIG family of ARF-GEFs (with the exception of GBF1). It does not affect the function of other ARF-GEFs from the Cytohesin family or others, which are thought to be located at the plasma membrane, as opposed to the BIG GEFs which are believed to be

Golgi-based (Donaldson and Jackson, 2000). To my knowledge, there is no available information on the location of these big ARF-GEFs in neutrophils.

In spite of these limitations, directional migration of neutrophils can be impaired by the use of BFA to the same extent as wortmannin. This would imply that if inhibition of the nucleotide-exchange reaction of ARF by ARF-GEFs and hence its activation provokes an arrest in the directional migration of these leukocytes, ARF proteins must be involved in the control of some aspect of the process. ARF might have a very specific function, as Rac and Cdc42 have (Srinivasan, 2003), and the coordinated activation and regulation of all these different GTPases will translate in the chemotactic movement of the cell. This assay might help to reveal the role of a completely disregarded albeit vital group of small G proteins involved in vesicle trafficking and actin regulation, in processes such as leukocyte chemotaxis.

There is previous evidence of ARF proteins being involved in motility of cells. For instance, ARF6 and its exchange factor ARNO have been implicated in epithelial cell scattering. Wounding or cell stimulation by HGF (scatter factor) triggers the activation of the ARF-GEF ARNO, resulting in the activation of both ARF and Rac (Turner and Brown, 2001). In other cases, ARF6 has been implicated in the formation of lamallipodia-like membrane protrusions, possibly through the ability of ARF6 to facilitate recruitment of active Rac to the plasma membrane (Radhakrishna *et al.*, 1999; Boshans *et al.*, 2000) and in other cases independently of Rac (Honda *et al.*, 1999). In macrophages, intact ARF6 function is required for coupling activated Rac to one of several effector pathways and suggests that a principal function of ARF6 is to coordinate Rac activation with plasma membrane-based protrusive events (Zhang Q. *et al.*, 1999). Expression of

a dominant negative mutant of ARF6 in HeLa cells has been shown to inhibit cell spreading (Song *et al.*, 1998).

From this set of experiments it can be deduced that ARF proteins could be directly involved in the regulation of the activity of at least PI3Kγ. This interaction seems to be restricted to the monomeric form of the enzyme. If proven to take place *in vivo*, ARF could conceivably be responsible for one of the two distinctive mechanisms for p110γ-activation that may occur in cells.

One would be the classic sequence of events: activation of a GPCR, GTP-loading of Gα and dissociation of Gβγ, recruitment of p101/p110γ via binding of the complex to Gβγ (primarily through p101) and enhanced production of $PI(3,4,5)P_3$.

As already noted, p101 is responsible for the recruitment of p110γ to the plasma membrane upon stimulation, which is equivalent to the function of p85 in Class IA enzymes. Brock and colleagues (2003) have shown that p110γ is not capable of binding to membranes unless p101 binds first to it and consequently brings it to the plasma membrane where it can be activated by Gβγ subunits and colocalise with its substrate. Gβγ subunits by themselves are not capable of recruiting p110γ probably because their interaction is weak, as opposed to that of Gβγ and p101 (Stephens *et al.*, 1997; Krugmann *et al.*, 1999; Maier *et al.*, 1999). They also showed that by mutating p110γ and including, for instance, an isoprenylation signal in its sequence, they could relocate the otherwise cytosolic monomeric p110γ to membranes where it is activated by Gβγ, even in the absence of p101. What is clear then is that forcing membrane localisation is sufficient to activate this enzyme but it is not clear how biologically relevant this is. Does p110γ occur in cells in the absence of p101? In the case of Class IA enzymes, p85

stabilises the catalytic subunit (Yu *et al.*, 1998) and so it is assumed that Class IA p110 subunits are only found together with their regulatory pairs. Conversely, p110γ seems to also occur in the absence of its regulatory counterpart (Baier *et al.*, 1999), and in fact p110γ is stable monomerically in cells whereas p101 is not (unless p110γ is present) and seems to be degraded rapidly (Brock *et al.*, 2003). Taken together, these data could grant the possibility of the existence of p110γ as a monomer, even if only transiently, and that it would be capable of functioning in cells apart from its regulatory subunit. This might provide an alternative mechanism of recruiting the catalytic subunit to membranes where its substrate is found. Indeed, there are reports that point at the downstream regulation of GPCR-dependent signal transduction pathways in cells by monomeric p110γ (Lopez-Ilasaca *et al.*, 1997, 1998; Bondeva *et al.*, 1998; Murga *et al.*, 1998; Baier *et al.*, 1999).

The second mechanism would therefore be the recruitment of p110γ independently of p101. Different mechanisms can be envisaged. As previously mentioned, *in vitro* evidence points at a possible anionic lipid-dependent association of p110γ to membranes, especially $PI(4,5)P_2$, PA and PS through a phospholipid binding domain (Kirsch *et al.*, 2001). Conversely, like all other PI3Ks, p110γ binds and is directly activated by GTP-bound Ras (Pacold *et al.*, 2000). GTP-Ras cannot recruit p110γ to membranes and binding of the enzyme to lipids seems to be an *in vitro* mechanism not reported *in vivo* and not observed when p110γ is overexpressed. As a result these two mechanisms could assist but not fully explain p101-independent p110γ-recruitment. The activation of ARF proteins could represent a additional means of tethering p110γ to the plasma membrane through direct binding and catalytic activation of the monomeric lipid kinase, although this would only be part of the mechanism of ARF activation.

The cellular control of the performance of ARF proteins is tightly dependent on PI3Ks and their lipid products. Several ARF-GEFs and ARF-GAPs possess PH domains that bind to $PI(3,4,5)P_3$, as well as other polyphosphoinositides such as $PI(3,4)P_2$ or $PI(4,5)P_2$, or phospholipids such as PA (Randazzo *et al.*, 2000). In many cases but not all, it has been demonstrated that the activation of ARF proteins via their GEF is dependent on PI3K activity (Klarlund *et al.*, 1998; Langille *et al.*, 1999; Venkateswarlu and Cullen, 2000; Bourgoin *et al.*, 2002). Also ARF inactivation is sensitive to PI3K activity (Vitale *et al.*, 2000; Miura *et al.*, 2002; Krugmann *et al.*, 2002; Venkateswarlu *et al.*, 2004). Indeed, readdition of p110γ together with ARF proteins and GTPγS into permeabilised HL60 cells caused a clear increase in the levels of PIP_2 as well as a rise in PIP_3. This is a clear demonstration of an improvement in the performance of the reintroduced ARF proteins conceivably through the activation of ARF-GEFs via association with the newly sythesised PIP_3. This cascade of events results, in all probability, in a better activation of PI(4)P5Ks, amongst other ARF effectors, and the consequent boost in $PI(4,5)P_2$ generation.

In parallel to what happens with GEFs for the Rho family of small G proteins, where disassociation of Gβγ subunits provokes their activation through $PI(3,4,5)P_3$ produced by PI3Kγ, tyrosine kinase-dependent phosphorylation of the GEFs or through downstream activation of Class I PI3K enzymes giving a positive feedback loop that further increases the levels of $PI(3,4,5)P_3$ produced, so it could happen with ARF-GEFs.

In conclusion, even though still unclear and with abundant questions and paradoxes to answer, it seems likely that ARF proteins are directly involved in PI3K metabolism. Extensive work needs to be done to clarify the remaining

questions and to assign correct functions and interrelation roles to these group of small G proteins within the already extremely intricate PI3K-metabolism field. It is striking that the involvement of these GTPases has been overlooked for so long when they are so closely related, functionally and metabolically, to all other constituents of the PI3K signalling cascades.

Chapter Seven

General Discussion

The principal theme of this thesis has been the study of the regulation of two of the enzymes involved in the synthesis of polyphosphoinositides, namely, Type I PI(4)P5K and consequently its product PI(4,5)P_2, and a Class I PI3K and its product PI(3,4,5)P_3. More precisely, I have attempted to characterise the influence of members of the ADP-ribosylation factor family of small G proteins, PKC and certain lipids on these kinases. Initially, an in-depth study of the preference of Type I PI(4)P5K isoforms for different isoforms of ARFs, the need for post-translational modification of the GTPases and a direct proof of protein-protein interaction was attempted. For this matter, *in vitro*, permeabilisation/ reconstitution in HL60 cells and direct protein-protein interaction pull-down assays were used to try to establish a strong basis from which to draw conclusions by using diverse but complimentary techniques. Furthermore, based on a bioinformatic analysis of the sequence of Type I PI(4)P5K and the prediction of the existence of a potential phosphorylation site for cPKCs, detection of such protein-modifying reactions *in vitro* was attempted but failed to provide conclusive results. Nonetheless, during the course of these experiments I discovered the overlooked stimulation of PI(4)P5K-activity caused by an important second messenger, diacylglycerol. Finally and completely accidentally, evidence of a possible novel control mechanism of PI(3,4,5)P_3-generation was found and further characterisation was attempted. This chapter will summarise the new findings, including their originality, and discuss their implications and contributions to the overall picture of polyphosphoinositide metabolism.

The data presented in this thesis indeed confirmed the well-established view that N-terminal myristoylation of ARF proteins plays a crucial role in ARF-function. More accurately, it demonstrates the requirement for this post-translational modification for the correct membrane-recruitment of the GTPase together with its effectors and subsequent stimulation on the activity of these enzymes. With the apparent exception of the plasma-membrane located isoform (murine) PI(4)P5K Iβ, which seemed to be less strict in its necessity for this lipid-modification and even GTP-dependent activation, solely in combination with ARF6, the control of all other isoforms of this particular lipid kinase by ARF were absolutely dependent on the presence of the myristate group. Likewise, the activation of Class IB PI3K as deduced from the permeabilised-cell assays, was also contingent on the presence of the N-terminal myristate.

Even more remarkable is the constant effect of nonmyristoylated forms of the protein under some of the conditions regarding their nucleotide-dependent activation. Recurrently, nonmyristoylated ARF proteins seemed to cause stimulation on its effector proteins in the absence of GTP to a higher extent than in conjunction with the nucleotide. This behaviour, as well as unpredicted, is usually thought to be completely unproductive in the normal function of ARF proteins. However, the effect was seen *in vitro* exclusively at high concentrations of ARF and at 37°C, and at more standard physiological concentrations in permeabilised cells, with both lipid kinases under study. Moreover, in biological terms, this scenario is artefactual as ARF proteins are always myristoylated. However, it is an extremely important insight that needs to be considered when judging or reviewing the information obtained in all of those studies that routinely use partially myristoylated populations of ARFs.

Furthermore, the use of fully-myristoylated ARFs made it possible to show a clear-cut difference between ARF6 and ARF1 in PI(4)P5K regulation. This is the first time a rigorous side-by-side assessment of the ARF isoform selectivity of PI(4)P5Ks has been achieved and is the direct result of avoiding partially myristoylated preparations of the GTPase. A wide range of approaches was used throughout this thesis in order to allow the evaluation of potential artefacts e.g. differences in the efficiency of GTP-loading or membrane-tethering as causal determinants of the improved activation of PI(4)P5K by ARF6 versus ARF1. As a result this investigation conclusively points to this isoform as the favoured activator of PI(4)P5K. Further support for this preference comes from the comparative use of the same fully-myristoylated ARF isoforms in the activation of p110γ in this same thesis and by studies of PLD by others. I found no significant preference between ARFs 1 and 6 for p110γ and in additional studies carried out in our lab on PLD (Ha, 2003), conversely it was established that ARF1 was the preferred activator using the same or identical preparation of protein.

The only problematic data was thrown-up while assessing the protein-protein interaction between PI(4)P5K and the two ARFs 1 and 6 along with their mutants. In this case, the preference for ARF6 was suppressed, and in activity terms it was inverted. Due to the more extensive persistent preference for ARF6 in all other approaches, including cell-free and cell-based assays which could be considered to be close to *in vivo* conditions, it seems wise to assume that in the case of the coprecipitation assay the discrepancy is an artefactual consequence of the overexpression system utilised to determine the existence of interaction, and/or the use of mutants. Further experimentation using a co-expression system (which was attempted but with no success due to technical difficulties) or co-

precipitation of endogenous proteins should help clarify this paradox and perhaps reveal an interaction more in accord with the rest of data.

Another original finding presented in this thesis is the existence of activation of PI(4)P5Ks by diacylglycerol. Although this discovery might seem to be of little immediate significance, for it might just be yet another lipid of the many that influence the activity of a wide range of enzymes *in vitro*, it must be remembered that DAG is generated by the direct degradation of $PI(4,5)P_2$. Besides, much work (and much presumption) has been made on the activation of this enzyme by an extremely closely related lipid, phosphatidic acid. This discovery should give plenty of reasons to rethink and redraw the pathways and interplay diagrams that define the control of the metabolism of this polyphosphoinositide and the multiple functions associated with it. Consequently, the results presented in many reports where PA is thought to be the direct activator of PI(4)P5K and thus its presence is used to explain certain behaviours and activities, need to also be reassessed. This finding situates DAG alongside PA in the control of the activation of PI(4)P5K, allowing for an even more powerful and straightforward activating machinery, as it elevates the activation of PI(4)P5K from being downstream of PLD (which is generally downstream of PLC) to being directly downstream of PLC as well. This activation by DAG is very much in line with standard, established biochemistry in that it is an example of product feedback, a well-demonstrated principle. Together with the confimed existence of a mechanism of activation of PI(4)P5K upon PKC activation, once again downstream of PLC activity, offers the possibility of strong collaborative reinforcement as part of a precise and potent regulatory mechanism. The demonstration of the existence of this dual, mutually

supportive feedback mechanism (direct activation by DAG and indirect via PKC) in living cells will be worth verifying but probably very difficult to demonstrate, because of the intricate interrelations of both PI(4)P5K and DAG with other enzymes and lipids, making it hard to isolate this process with certainty from the surrounding influences.

At the same time, exhaustive exploration of differences in the *in vitro* activation of PI(4)P5K by different lipids during this thesis calls for the careful consideration of conclusions drawn from these type of experiments using lipid metabolising enzymes, as factors such as the precise composition of substrate vesicles, which include the enclosure of carrier lipids and the molar ratios in use, the nature of the side chains of the lipids under study, the inclusion of detergents and the origin of the enzyme, can all alter the result and lead to divergent inferences unless careful comparative consideration of all these variables is undertaken.

Finally the striking novel discovery that ARF proteins could activate a specific PI3K (or inhibit both the β and γ isoforms in the presence of their regulatory subunits) was made completely fortuitously. ARF proteins have already been shown to activate some PI4Ks and PI(4)P5Ks, thus controlling all the previous steps necessary for the ultimate generation of the second messenger $PI(3,4,5)P_3$ under the control of Class I PI3Ks. Hence, the possibility of at least part of the $PI(3,4,5)P_3$-production being subject to ARF-regulation does not sound outlandish. Moreover, since the activity of ARF seems to be closely linked to the presence of this polyphosphoinositide through its GEFs, and as seen in the permeabilised-cell assays its own presence not only reinforces its generation in

the presence of ARF but also that of its precursor PI(4,5)P_2, it seems plausible that a set of interrelations link all these kinases together with ARF. This way, a precise polyphosphoinositide-generating mechanism could be created and hence the cell-physiological functions that are PI(3,4,5)P_3-dependent can be tightly managed and co-ordinated.

Further characterisation of this effect led to the finding that p110γ the only isoform of Class I PI3Ks whose activity can be stimulated by the presence of the small GTPase. However, it is still possible that other isoforms can also be influenced by ARF proteins, as the assay conditions might be optimised in order to fully assess the possibility of interaction. *In vitro* study of the activation of p110γ could not establish a difference between GDP- and GTP-loaded ARF, although this was probably due to the precise *in vitro* conditions, as in cell-based assays there was a clear preference for GTP analogues. On the other hand, the activation of p110γ by ARF was limited to its monomeric state as the presence of its regulatory subunit exerted the opposite effect, making ARF-driven recruitment of p110γ a conceivable alternative to the well-known p101-dependent tethering of the lipid kinase. However, if other adaptors for p110γ are discovered then the effects of ARFs on the new kinase heterodimers will need to be evaluated.

An interesting idea is that, ARF-dependent activation of p110γ, independent of p101 and therefore Gβγ dissociation, could situate monomeric PI3Kγ downstream of Class I PI3Ks. However, to my knowledge, there is no evidence of such an event occurring in cells.

The studies undertaken in this thesis have focused mainly on *in vitro* assessment of the activation of phosphoinositide kinases by different ARF

proteins and lipids. This is a solid foundation on which to build hypotheses regarding ARF regulation of phosphoinositide metabolism. However in physiological terms, there are limitations to the scope of these studies. In the case of the preference of phosphatidylinositol 4-phosphate 5-kinases for ARF6 over ARF1, although a clear biochemical difference has been shown in cell-free and broken cell systems, confirmation of this effect in intact cells needs to be obtained. Other factors such as the presence of GEFs/GAPs, the lipid composition of the membranes in which they act and other effectors and regulators might reveal that this natural preference is subject to further controls. In those cases where both ARF isoforms coincide spatially and temporally within a region of the cell, this preference might have sufficient weight to determine the preferential interaction of these isoforms with their different effectors, for instance ARF6 acting on PI(4)P5K while ARF1 acts on a different effector such as PLD. Alternatively, other factors that control the activity of the lipid kinases might overcome this natural preference and determine the real inclination within cells. In those cases where these isoforms do not colocalise in specific cellular regions, recruitment of the specific isoforms by GEFs or other mechanisms might be the real determinant of such activation, allowing for the possibility of ARF1 acting on PI(4)P5K regardless of its reduced effect if compared with ARF6.

Due to the high complexity of the cell environment, these simplified biochemical studies are a necessary first step towards the elucidation of the patterns of interaction of these proteins *in vivo*. Nonetheless, further work needs to focus on the confirmation of such natural preferences within cellular systems. So is also the case with regards to the activation of PI(4)P5K by DAG or the activation of monomeric p110γ by ARF proteins. Strong proofs are difficult to obtain in whole cells again because of the complexity of the system and also

because of redundancy in signalling pathways and so both approaches are mutually complimentary. For example biochemical studies can accurately guide the creation of effective genetic or pharmacological interventions.

Continuation of the work described here might focus on the following issues:

Confirmation of the preference of ARF6 over ARF1 on the activation of PI(4)P5Ks in cells. For this matter, different approaches could be used such as the use of RNAi. Elimination of a specific ARF isoform, such as ARF6, and measurement of the cellular levels of $PI(4,5)P_2$ or the influence of this exclusion in processes where ARF and PI(4)P5Ks are known to be involved, such as the formation of membrane ruffles or Golgi function, could give an idea of whether one isoform can efficiently substitute for the other or whether their cellular roles are clearly distinguished as well as which processes depend on which isoforms. Additionally, the same could be done with the different PI(4)P5K isoforms and combinations of the isoforms of the two proteins. $PI(4,5)P_2$ levels could be monitored using radioactive methods or a fluorescent probe, such as a GFP-fusion protein with the PH domain of $PLC\delta_1$ which specifically binds $PI(4,5)P_2$.

Another approach would be the use of knockout or knockdown mice where the exression of ARFs or PI(4)P5Ks could be eliminated/downregulated in order to examine the requirement for these enzymes in ARF1 or ARF6 dependent processes. PI(4)P 5-kinase α and γ knockout mice have already been produced and may be effective and valuable tools.

Indication of linkage between ARF and $p110\gamma$ could be studied by monitoring the increase in $PI(3,4,5)P_3$ generation in living cells when they are transfected with constitutively active or dominant negative ARF mutants.

Preliminary experiments indicate that cotransfection of ARF1(Q71L), p110γ and Grp1-PH domain-eGFP fusion protein (a fluorescent marker of $PI(3,4,5)P_3$ synthesis) produces a profound increase in the fluorescence of the plasma membrane.

Permeabilisation assays using cell types where p110γ is absent, such as HEK293 cells or other fibroblasts, could clarify whether the increase in PIP_3 levels registered after re-addition of ARF proteins are solely caused by p110γ activation or whether there is any input from the other isoforms.

Interaction studies by coexpression and coprecipitation of PI3Ks and ARF proteins in order to demonstrate a direct interaction between the two proteins would also be valuable and RNAi of ARF isoforms in cell types where p110γ function is well established, such as neutrophil migration or platelet aggregation, could help determine whether there is any involvement of ARF proteins in such processes.

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